University of Alberta

Sleep-like rhythmic alternations of brain state in the urethane-anesthetized animal

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Abstract

Although the induction of behavioral unconsciousness during sleep and general anesthesia has been shown to involve overlapping brain mechanisms, sleep involves cyclic fluctuations between different brain states known as active (paradoxical or REM) and quiet (slow-wave or nREM) sleep whereas all known general anesthetics induce a unitary slow-wave brain state. Here I report, for the first time, a spontaneous rhythmic alternation of brain state under urethane anesthesia that is comparable in its EEG components, time frame, physiological correlates, pharmacology, and ascending brainstem mechanisms to natural sleep state alternations. This suggests that urethane promotes a condition of behavioral unconsciousness which closely mimics the full spectrum of natural sleep. The use of urethane anesthesia as a model system will facilitate mechanistic studies into sleep-like brain states and their alternations and could also be exploited as a tool for the discovery of molecular targets to promote sleep without compromising state alternations.

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Chapter 1 – Introduction

The universality of sleep substantiates its importance. This is further highlighted by the fact that sleep-related problems have been reported as the second most frequent complaint doctors encounter (Mahowald & Schenck, 2005). As deprivation results in dysfunction, it is not surprising that these complaints reach the level of the physician. Treatment of these complaints is difficult because sleep itself, although seemingly simple, is in fact a highly complicated phenomenon.

In the early 1900's, it was presumed that sleep was a passive result of the inactivation of arousal systems that rendered the brain quiescent. However, the discovery of regular periods of wake-like brain activity patterns coupled with rapid-eye movements during sleep, precipitated renewed investigations into sleep. It is now known that normal electrical brain activity during mammalian sleep evolves through 5 distinct stages. The first four are collectively referred to as non-rapid-eye movement (nREM) sleep, and can be broken into stages 1&2, known as light sleep which emerge with sleep onset, and stages 3&4, known as slow-wave sleep (SWS). SWS precedes the fifth state, rapid-eye movement (REM) sleep, characterized by, as its name implies, rapid-eye movements, wake-like brain activity, and a loss of muscle tone (Aserinsky & Kleitman, 1953; Jouvet, 1969). With these findings, sleep research has expanded to include different brain states, transitions between states as well as the functional role of sleep within the context

of human existence. And while progress continues and many aspects of sleep are better understood, there are many complexities that remain elusive.

Basic characteristics of sleep

Correlates of the circadian sleep rhythm

Approximately one third of the average human life is spent sleeping, with the basic need for sleep occurring once every day. This drive can be deliberately overcome and, under these circumstances, the arrival of a new day will be associated with a renewed sense of arousal. Consequently, however, sleep need increases and lost sleep time will be compensated for in subsequent sleep periods, all suggesting that sleep and sleep drive are physiologically regulated (Saper, Scammell, & Lu, 2005). If external cues, such as light, are removed, the drive to sleep once in a 24-hour period remains constant, indicating that this regulation is actually internal (Zee & Manthena, 2007). This 24-hour sleep-wake process is physiologically known as a circadian rhythm; the drive to sleep is produced internally, however, environmental factors, such as light or a social situation, can have modulatory affects. When the drive to sleep occurs in an environment and at a time that is conducive to sleep, muscle tone begins to decline, attention is lost, and slow asynchronous eye movements begin to take place, all signaling the onset of sleep.

Correlates of the REM/nREM sleep cycle

The onset of sleep is associated with the emergence of light sleep, which gradually deepens to SWS before an abrupt transition into REM. The offset of

REM is concomitant with the re-emergence of light sleep. These alternations form an ultradian rhythm known as the REM/nREM sleep cycle. In humans, it is approximately 90 minutes in duration and occurs approximately 5 times in one night, where cycles earlier in the night are composed mostly of SWS and later cycles are composed mostly of REM sleep (Siegel, 2004).

Characteristics of nREM sleep

As previously mentioned, nREM sleep is composed of four stages, loosely grouped into light sleep and SWS. With the onset of light sleep, wake associated rhythms such as alpha (8-13Hz) are replaced with lower-voltage, mixed frequency patterns. Stage 2 sleep, in particular, is associated with increased power within the spindle (7-14Hz) bandwidth. In the hippocampus, there is also an increase in power in lower frequency bandwidths as it displays large-amplitude irregular activity. Power in the delta (1-4Hz), and in particular, the lower frequencies of this bandwidth, increases as sleep deepens into stages 3&4, whereupon both cortex and hippocampus demonstrate a slow wave oscillation of approximately 1Hz (Wolansky, Clement, Peters, Palczak, & Dickson, 2006).

The progression from stage 1 to stage 4 sleep involves the slow (<1Hz) oscillation as well as interactions between the cortex and thalamus. The slow (<1Hz) oscillation involves alternations of cortical membrane potentials between hyperpolarized (down) and depolarized (up) states. An abrupt shift to a depolarized state appears in the cortical electroencephalogram (EEG) along with a surface positive deflection known as the K-complex (KC). Via cortico-thalamic projections, KCs elicit rhythmic burst firing within the thalamus which is

subsequently reflected as a sleep spindle in the cortex, an envelope of oscillations in the 7-14Hz bandwidth that lasts approximately 1 second and that forms the tail of the KC. (Amzica & Steriade, 2002; Steriade & Amzica, 1998). As sleep deepens, the frequency of the transitions from down to up states increases from approximately 0.5 to 0.9Hz and the rhythms following the KC become slower. Eventually, a KC occurs once every second, which presents electrographically as the slow oscillation. Following the end of stage 4, there is an ascent back through each of these stages, leading into REM sleep.

Characteristics of REM sleep

The transition into REM sleep is associated with the emergence of wake-like EEG activity patterns, such as low-voltage fast activity in the cortex and rhythms in the theta (3-12Hz) frequency bandwidth in the hippocampus. These active patterns are coupled with peripheral atonia and behavioural unresponsiveness, which is why REM sleep is also known as paradoxical sleep (Jouvet, 1969). The REM state is also associated with increases in heart rate as well as irregularities in respiration rate (Aserinsky, 1965; Zemaityte, Varoneckas, & Sokolov, 1984), and with the conscious experience of dreaming (Hobson, 2005).

The presence of wake-like activity patterns in the cortex during REM sleep suggests an overlap in the neurotransmitter systems involved in these two conditions, however there are subtle differences. In general, the production of cortical and hippocampal activation during the waking state is dependent on several more neurotransmitter systems in comparison to their activation during REM sleep (Jones, 2003). For example, the hippocampal theta rhythm, which in

REM sleep is associated with immobility, is dependent solely on the excitatory neurotransmitter acetylcholine (ACh) (Vanderwolf, Kramis, & Robinson, 1977). During wakefulness, however, this rhythm is dependent on both monoamines and ACh and is associated with voluntary behaviours, such as changes in posture and running.

Neurotransmitter systems involved in sleep and wake states

Contrary to theories stipulating that the brain is inactive during sleep, the expression of arousal as well as sleep states is dependent upon activity and interactions of forebrain, midbrain and brainstem structures. The brainstem nuclei involved in arousal include the serotonergic raphé nucleus, the noradrenergic locus coeruleus, and the cholinergic pontine nuclei. While both serotonergic and noradrenergic nuclei are most active during wake, less active during nREM sleep and quiet in REM, cholinergic pontine nuclei are highly active in both wake and REM and quiet in nREM. Additionally, midbrain structures contributing to arousal include the histaminergic tuberomammillary nuclei and orexinergic neurons in lateral hypothalamus, both of which are most active in waking. Furthermore, the cholinergic basal forebrain nuclei also contribute to waking as well as REM sleep (Jones, 2003; Jones, 2005). In addition to these neurotransmitter systems, GABAergic nuclei in the anterior hypothalamus, which have outputs to all major arousing nuclei in the brainstem and hypothalamus, are most active during sleep (Saper et al., 2005). A basic schematic of these nuclei and their projections is shown in Figure 1.

Figure 1. Neurotransmitter systems involved in waking and sleep states.

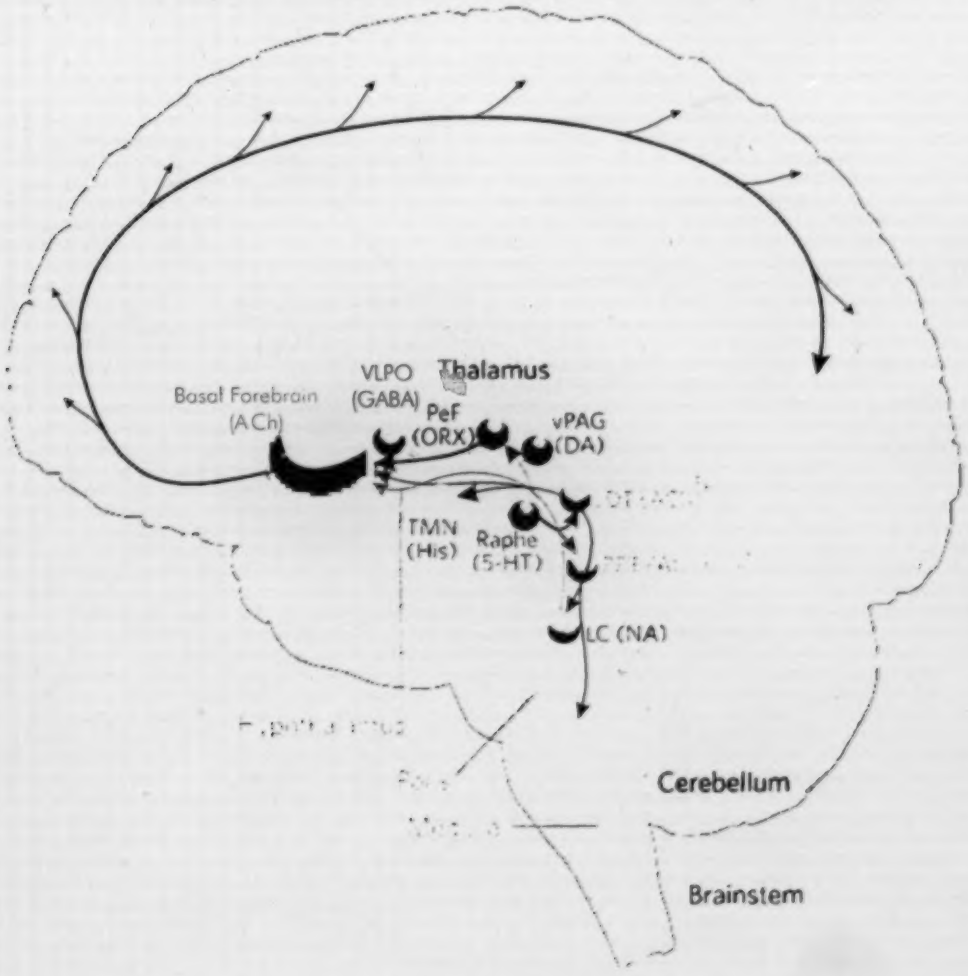

Figure 1. Neurotransmitter systems involved in waking and sleep states.

Cholinergic pontine (orange) nuclei project within the brainstem to the locus coeruleus, as well as towards the spinal cord where, during REM sleep, they induce atonia. They also project to midbrain nuclei, including the hypothalamus, as well as basal forebrain regions. Noradrenergic neurons in the locus coeruleus (blue) project to midbrain areas including the hypothalamus, and forebrain areas including the basal forebrain and the neocortex. Serotonergic raphe neurons (red) have diffuse projections to the cortex as well as to pontine nuclei. Midbrain hypothalamic nuclei, including the histaminergic tuberomammillary nucleus (yellow) and the orexinergic (green) lateral hypothalamus have diffuse projections to the cortex. Orexinergic neurons also excite other wake-promoting nuclei, including the locus coeruleus, the basal forebrain and the tuberommammillary nucleus. Cholinergic nuclei of the basal forebrain are innervated by pontine cholinergic nuclei, the locus coeruleus, tuberomammillary nucleus and lateral hypothalamus and have diffuse excitatory projections to the neocortex. Schematic has been simplified so as to include neurotransmission important for activation during sleep.

Adapted from: Saper, C. B., Scammell, T. E., & Lu, J. (2005). Hypothalamic regulation of sleep and circadian rhythms. *Nature, 437*(7063), 1257-1263.
Jones, B. E. (2003). Arousal systems. *Frontiers in bioscience [computer file]: a journal and virtual library, 8*, s438-51.

Regulation of sleep

The basic mechanisms of sleep onset

Sleep was originally thought to be a passive result of inactivation of waking systems. This theory, however, was discarded when experiments that involved transections of the brainstem behind the oral pontine regions resulted in insomnia, suggesting that brainstem structures posterior to this transection played an active role in the generation of sleep (Jouvet, 1962; Siegel, 2004 as cited in Kryger, Roth, & Dement, 2005). Furthermore, midbrain lesions, particularly of the anterior hypothalamic and preoptic nuclei, resulted in disordered sleep while stimulation of basal forebrain structures elicited activated patterns followed by the onset of sleep (Nauta, 1946; Steininger, Alam, Gong, Szymusiak, & McGinty, 1999; Sterman & Clemente, 1962a; Sterman & Clemente, 1962b). Taken together, these data demonstrate that the onset of sleep is not localized to one specific area of the brain, but rather, it involves interactions between several loci. Very briefly, hypothalamic structures, which are sensitive to external cues such as light, body temperature, and satiety, as well as internal circadian cues, change the their firing patterns; areas such as the preoptic nucleus increase firing, and posterior and lateral regions including the tuberomammillary nucleus, decrease firing (Gvilia, Xu, McGinty, & Szymusiak, 2006; Lu et al., 2002; Saper et al., 2005; Steininger et al., 1999; Szymusiak, Alam, Steininger, & McGinty, 1998). Also, wakefulness results in an accumulation of adenosine, a byproduct of neural metabolism, which inhibits the arousing cholinergic basal forebrain structures (Porkka-Heiskanen et al., 1997). These areas have projections to arousing nuclei in the brainstem,

including the raphé nucleus and the locus coeruleus, which respond by becoming less active (Lu, Sherman, Devor, & Saper, 2006). Moreover, other brainstem nuclei, including the pontine tegmentum, become less excitable in response to a decline in sensory input as well as the accumulation of adenosine (Porkka-Heiskanen et al., 1997; Rainnie, Grunze, McCarley, & Greene, 1994). When all these factors come together, the brain transitions into light sleep.

The potential mechanisms of alternations between sleep states

The fact that there are generally two distinct stages of sleep (i.e., REM and nREM) inherently means that by some mechanism, alternations between states must occur. Areas involved in state alternations became apparent in 1962 when Jouvet, a French experimenter, transected the brainstem at a level above the pons, which resulted in an absence of REM sleep in the forebrain structures. However, caudal to the lesion, generation of REM sleep remained present as evidenced by periods of peripheral atonia coupled with electrical spikes, typically associated with rapid-eye movements in REM sleep (Jouvet, 1962 as cited in Kryger et al., 2005 and Steriade & McCarley, 1990). If the transection occurred just caudal to the pons, REM sleep could be observed in the forebrain, but not in the medulla. This strongly implicated pontine nuclei in the control and transition into the REM sleep state (Jouvet, 1962; Siegel, 2006).

Increased discharge rates of neurons within specific pontine nuclei during REM sleep supported this hypothesis, and further led to the proposal of the reciprocal interaction model (Fig. 2), which, until recently, was widely accepted (Hobson, McCarley, & Wyzinski, 1975). This model involved inhibition of the

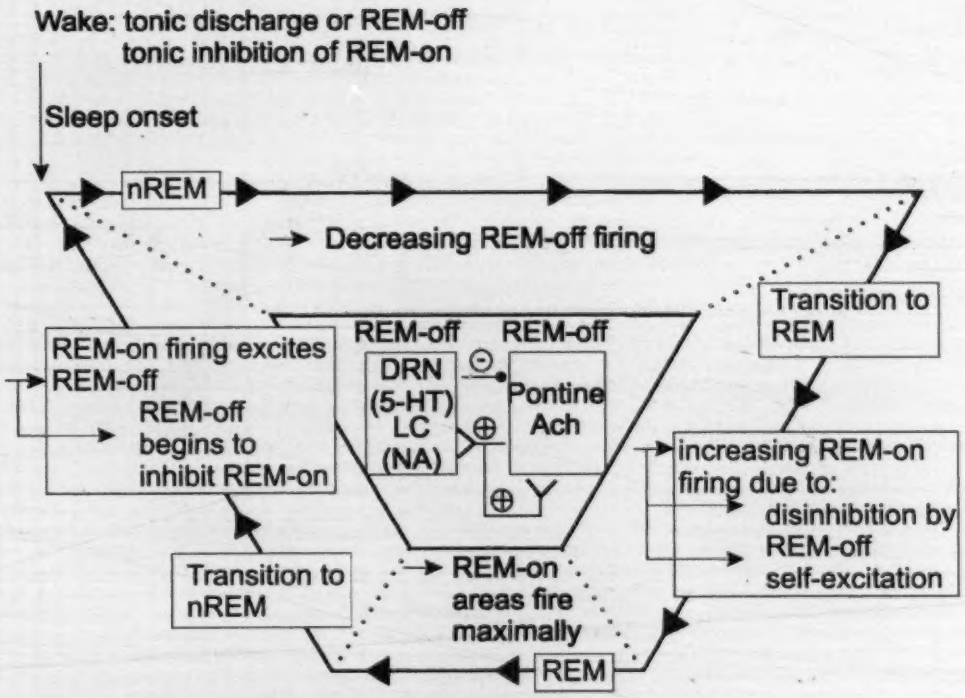

Figure 2. The reciprocal interaction model.

REM-off nuclei in the brainstem include the serotonergic raphe nucleus and noradrenergic locus coeruleus which inhibit the pontine REM-on nuclei. The pontine REM-on nuclei excite both itself and the REM-off nuclei. As sleep sets in (top left hand corner) and progresses through the stages of nREM sleep, the tonic discharge of the REM-off area begins to fade, relieving the strong tonic inhibition of REM-on area. This triggers a transition into REM sleep, which culminates when REM-on areas firing maximally during the peak of the REM sleep state. The maximal firing of the REM-on areas excites the REM-off areas, increasing in the inhibition to the REM-on areas, and spurring a transition back into nREM.

Hobson, J. A., McCarley, R. W., & Wyzinski, P. W. (1975). Sleep cycle oscillation: Reciprocal discharge by two brainstem neuronal groups. *Science (New York, N.Y.), 189*(4196), 55-58.

cholinergic pontine nuclei (REM-on) by the aminergic locus coeruleus and raphé nuclei (REM-off). With transitions into sleep and the progression through nREM, aminergic cells would decrease their firing rate, effectively removing inhibition from the pontine cholinergic nucleus. Eventually, a transition to REM would occur and cholinergic neurons fire maximally. These cells, while producing self-excitation, also re-excited the aminergic cells which in turn began to inhibit cholinergic nuclei until the balance would shift, resulting in a transition back into nREM sleep (Hobson et al., 1975; Merica & Fortune, 2004).

Recently, however, this model has been challenged as new areas of the brainstem have been implicated in the regulation of REM sleep. A study was conducted using knowledge of hypothalamic areas, specifically the excitatory orexinergic cells, known to contribute to the regulation of REM sleep as they are absent in individuals with narcolepsy, a sleep disorder which involves abrupt, involuntary transitions from the waking state directly into REM sleep (Chemelli et al., 1999), and preoptic neurons are inhibitory and fire during REM sleep. This study exploited the fact that, unlike preoptic neurons, orexinergic neurons are excitatory and cease firing during REM sleep (Jones, 2005; Lu et al., 2002; Steininger et al., 1999). It was, therefore, presumed that the area of the brainstem where these two groups converge would be a REM-off site, also inactive during REM sleep. Simultaneous anterograde tracing demonstrated that these two groups innervate overlapping areas within the mesopontine tegmentum, specifically, the lateral pontine tegmentum and the venterolateral periaquaductal grey. Cell specific lesions of this REM-off site produced large increases in REM sleep,

confirming their role in the control of REM sleep. Furthermore, it was shown that this site had reciprocal inhibitory connections with the REM-active areas of the adjacent mesopontine tegmentum, including the sublaterodorsal nucleus and the precoeruleus. Lesions to these sites resulted in marked decreases in REM sleep (Lu et al., 2006). These findings implicated that REM sleep was being regulated by areas that, in terms of the reciprocal interaction model, had previously been overlooked.

Although this study presents new and intriguing data, it was additionally reported that lesions (using ibotenic acid) to cholinergic cells within areas previously implicated in the REM/nREM sleep cycle resulted in no alteration of REM sleep (Lu et al., 2006). Taking into consideration the previous model, as well as years of research, connecting ACh to both state transitions and the maintenance of REM sleep, this is a surprising finding (Hobson et al., 1975; Jones, 2003; Merica & Fortune, 2004). Thus, there remains a lack of clarity surrounding the loci and mechanisms of the REM/nREM state transitions.

Evidence supporting the potential underlying functions of individual sleep states

Taken together, the unique states of sleep, the regular variation of state, and the transitions themselves frame why sleep is so intriguing. It has affirmed sleep to be an active and not a passive process which raises the question: What is the purpose of sleep and further, of individual sleep states?

The idea that REM sleep serves a specific purpose was supported by the finding that following selective REM sleep deprivation there were increased attempts to enter the REM state indicating that this state was under a form of homeostatic regulation (Rechtschaffen, Bergmann, Everson, Kushida, & Gilliland, 2002). Furthermore, many of the effects of total sleep deprivation, including skin lesions, weight loss, increased plasma norepinephrine, and a decrease in body temperature could also be elicited with selective REM sleep deprivation (Rechtschaffen et al., 2002). However, some of these effects, including the effects on body temperature, were not consistent with selective deprivation of nREM sleep. This highlighted that individual sleep states serve overlapping but also unique purposes.

Subsequent experiments have implicated sleep states in the consolidation of new memories (Stickgold, 2005; Walker & Stickgold, 2006). Briefly, evidence has accrued for the improvement on non-declarative memory tasks during sleep, with tasks like texture discrimination relying more heavily on REM sleep and stages 3&4, and motor sequence learning relying on stage 2 sleep, where spindles appear (Walker & Stickgold, 2006). Furthermore, there is increasing evidence amounting to the fact that rhythms elicited in sleep states, including the slow (<1Hz) oscillation, sleep spindles, theta in the hippocampus, and cortical slow-wave are conducive to memory formation (Born, Rasch, & Gais, 2006; Eschenko, Molle, Born, & Sara, 2006; Fogel & Smith, 2006; Huber, Ghilardi, Massimini, & Tononi, 2004; Marshall, Helgadottir, Molle, & Born, 2006; Rauchs, Desgranges, Foret, & Eustache, 2005).

There is, however, a considerable amount of debate in terms of the sleep-memory consolidation hypothesis. For example, some authors believe that SWS is required for the consolidation of declarative memories, as oppose to non-declarative tasks. There is even debate over whether consolidation of non-declarative memories relies more heavily on REM sleep or on stages 1&2 (Rauchs et al., 2005). In fact, there remains considerable debate over whether sleep does, indeed, contribute to the consolidation of new memories at all (Maquet, 2001; Siegel, 2001; Vertes, 2004). Ultimately, there is a large amount of ambiguity associated with sleep related advancements in research.

Limitations and challenges in sleep research

Making headway in sleep research is often hindered both functionally and ethically. These studies often require direct manipulation of deep brain structures while observing gross electrical changes, ideally in the absence of concomitant behavioural changes. Sleep research in humans is limited to a sleep laboratory where several superficial EEG electrodes are attached to the scalp, as well as several additional peripheral electrodes which acquire physiological signals, such as the electroocculogram (EOG) and electromyogram (EMG). This all occurs while the individual attempts to sleep in an unfamiliar bed located in an unfamiliar context. The use of laboratory animals, in many ways, allows the evasion of several of these confounds, via implantation of tools, such as electrodes, stimulators, or cannulae, which are targeted at recording from or manipulating deeper brain structures. However, this technique reaches limits as

well, as certain manipulations may either disrupt sleep or induce pain and severe discomfort, making them unsuitable and potentially unethical in a behaving animal.

The role of anesthetics in sleep research

The introduction of anesthetics has been beneficial to neurophysiological studies in general as it circumvents both ethical concerns and issues of behavioural stability. Insofar as sleep, it has also been beneficial since there are extensive similarities between the sleeping animal and the anesthetized animal, both being characterized by reduced motor activity and responsiveness and exhibition of brain wave patterns that are absent in wakefulness (Tung & Mendelson, 2004). Sleep is, in fact, the most commonly used metaphor for anesthesia (Shafer, 1995). Moreover, recent research has unveiled both functional and mechanistic similarities across sleep and anesthesia. It has been shown that general anesthetics, including propofol and pentobarbital, can induce their sedative affects via inhibition of the arousing histaminergic neurons of the tuberomammillary nucleus (Nelson et al., 2002). Propofol has also been shown to induce sedation when infused directly into the preoptic area (Tung, Bluhm, & Mendelson, 2001). Furthermore, it has been demonstrated that following recovery from 12 hours of propofol sedation (which overlapped with their normal sleep phase), animals did not display behavioural symptoms of sleep deprivation (Tung, Lynch, & Mendelson, 2001). Moreover, following sleep deprivation, comparisons of groups of animals either subjected to propofol anesthesia or allowed to sleep ad

lib, did not show differences in correlates of recovery, including total sleep time and proportion of sleep spent in different states, in subsequent sleeps. (Tung, Bergmann, Herrera, Cao, & Mendelson, 2004). All these lines of evidence suggest that anesthetics are useful tools in terms of sleep research.

Despite the similarities between sleep and anesthesia, however, there are fundamental differences that segregate the two. Whereas sleep is physiologically regulated and a reversible state, anesthesia is a chemically induced state from which an animal cannot be roused until the anesthetic has been metabolized. And while anesthetics can be used in modeling nREM states, these brain states are unitary and state alternations are, therefore, absent. Ultimately this poses obstacles in investigations of REM sleep and in the mechanisms of REM/nREM state transitions (Tung & Mendelson, 2004).

Basic characteristics and usages of the veterinary anesthetic urethane

Urethane, also known as ethyl carbamate, is a commonly used veterinary anesthetic (Koblin, 2002). It was originally used as a cancer fighting agent, however, with the discovery of its toxicity, this usage became limited. Regardless, trace amounts of urethane can be found in alcoholic beverages, soya sauce, and breads (Koblin, 2002). Despite the prevalence of its use as a veterinary anesthetic for surgical experiments, the anesthetic mechanisms of urethane are largely unknown. Regardless, what is understood of the anesthetic mechanisms, which includes a recent study which suggested that urethane induces an increase in a

leak conductance, resulting in a decrease in excitability, the mechanism of urethane sets it apart from other general anesthetics, most of which produce their sedative affects by agonizing inhibitory neurotransmission(Sceniak & MacIver, 2006). The metabolism of urethane also differs from other anesthetics in that it occurs slowly, resulting in an anesthetic state that lasts for long durations (several hours). The slow rate of metabolism makes the use of urethane advantageous in comparison to other anesthetics, and further has facilitated its use as a model for both slow wave states, including spindle generation, and REM sleep via conjunction with cholinomimetics (Contreras, Destexhe, Sejnowski, & Steriade, 1997; Horner & Kubin, 1999; Kinney, Vogel, & Feng, 1998; Lee, Kim, & Shin, 2004; Steriade, Nunez, & Amzica, 1993).

This project discusses the finding of spontaneous, rhythmic alternations of brain state under urethane anesthesia in the rat. It investigates the temporal and physiological correlates of these alternations, directly or indirectly comparing them to those in natural sleep. It further evaluates the neurochemical and neuroanatomical basis of states and alternations, making reference to current knowledge of natural sleep. It concludes by discussing the potential of the urethane anesthetized animal as a model for the full spectrum of natural sleep.

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Chapter 2 – Sleep-like rhythmic alternations of brain state in the urethane-anesthetized animal

Introduction

Sleep is a condition of altered consciousness in which there is a reduction of sensory awareness, behavioral output and metabolic activity. Sleep is expressed as a circadian rhythm under neural control and appears to be essential for survival (Rechtschaffen, Bergmann, Everson, Kushida, & Gilliland, 2002). Although sleep takes up a significant portion of our biological existence, its functional purpose (both ecological and physiological) remains unclear. One of the fundamental mysteries of sleep is the production of brain state alternations; the REM/nREM cycle.

Sleep in mammals and birds involves two main stages as measured by EEG recordings: 1) quiet, slow-wave, or nREM sleep, which is characterized by large-amplitude and slow cortical rhythms; and 2) active or REM sleep, which is characterized by low-amplitude and faster cortical rhythms. The latter stage is also known as paradoxical sleep since the EEG patterns are not unlike those present during alert waking. Alternations between nREM and REM sleep occur at regular intervals throughout a contiguous sleep episode. The functional relevance of these stages and their alternations is unknown, although depriving subjects of either stage can induce detrimental effects independent of sleep loss itself, and a rebound amount of time spent in the deprived state in subsequent sleep episodes (Ferrara, De Gennaro, & Bertini, 1999; Ocampo-Garces, Molina, Rodriguez, &

Vivaldi, 2000; Rechtschaffen, Bergmann, Gilliland, & Bauer, 1999; Rechtschaffen & Bergmann, 2002; Rechtschaffen et al., 2002).

Although progress towards an understanding of 1) the mechanisms, and 2) the functional relevance of state dependent patterns of brain activity and their alternations in sleep has certainly been made since their discovery in 1953 (Aserinsky & Kleitman, 1953), research in the field has been made difficult by the lack of a model of sleep state alternations other than sleep itself. For certain experimental paradigms the naturally sleeping animal presents both technical and ethical obstacles. This has undoubtedly limited our scientific progress in this field. The most common model for sleep has been anesthesia, which produces a behavioral condition not unlike natural sleep (Nelson, Franks, & Maze, 2004). In fact, "sleep" is the most common metaphor or analogy used for anesthesia by physicians and laypersons alike (Shafer, 1995). The similarities between sleep and anesthesia include: a subjective loss of consciousness, reduced sensory awareness, and a reduction in behavioral responsiveness. Moreover, recent research has provided evidence that there is a considerable overlap in the physiological mechanisms of anesthesia and the induction of the sleeping state (reviewed in Tung, Bergmann, Herrera, Cao, & Mendelson, 2004). Of course, differences between the two conditions are obvious, including the dependence of anesthesia on circulating levels of pharmacological agents, the inability to rouse anesthetized subjects and, perhaps most importantly, the lack of cyclical variability of brain states while under anesthesia.

Here I report the initial finding that rats anesthetized with urethane demonstrate spontaneous and cyclical alternations of brain state that resemble sleep state alternations in their EEG components. This study then evaluates the evolution, time frame, physiological correlates, pharmacology and dependence upon ascending neuromodulatory brain systems of the alternations of brain state in the urethane-anesthetized animal. I predict these investigations will yield similarity of this condition to the naturally sleeping animal. My results suggest that the pharmacological action of urethane in the brain closely mimics the physiological mechanism(s) for the maintenance of the natural sleeping state in rats and implicating this preparation as a valid model for the investigation of brain mechanisms giving rise to cyclical state alternations.

Materials and Methods.

Data were obtained from 94 male Sprague-Dawley rats weighing between 171.0 and 410.2g (262 ± 5.1). All methods used conformed to the guidelines established by the Canadian Council on Animal Care, the Society for Neuroscience, and were approved by the Biosciences Animal Policy and Welfare Committee of the University of Alberta.

Acute (urethane-anesthetized) preparation.

Anesthesia and surgery. Anesthesia was initially induced with gaseous isoflurane mixed with medical oxygen at a minimum alveolar concentration (MAC, the partial pressure of a vapour where 1.0 is the concentration of the substance that is necessary to induce immobility in 50% of subjects) of 4.0 in an

enclosed anesthetic chamber. Following loss of righting reflexes, they were maintained on isoflurane (2.0 to 2.5 MAC) via a nose cone and implanted with a jugular catheter. Isoflurane was discontinued and general anesthesia was achieved using slow intravenous (i.v.) administration of urethane (0.8g/ml; final dosage 1.75 ± 0.04g/kg). Body temperature was maintained at 37°C using a servo driven system connected to a heating pad and rectal probe (TR-100, Fine Sciences Tools; Vancouver, BC, Canada) for the remainder of the surgical and recording procedures. Level of anesthesia was assessed throughout the experiment by monitoring reflex withdrawal to a hindpaw pinch.

Stereotaxic procedures. Stereotaxic placement of indifferent, recording, and stimulating electrodes in addition to infusion cannulae was conducted using bregma as a coordinate landmark. Recording electrodes were constructed from Teflon-coated stainless steel wire (bare diameter 125µm: A-M Systems Inc.). These electrodes were aimed at the frontal (AP: +0.3; ML: ±1.0), and posterior (AP: -4.5, ML: ±1.5mm) neocortices, either in superficial or deep layers (DV: -0.1 to -0.25 or DV: -1.0 to -1.5mm, respectively) and the hippocampal fissure of the dorsal HPC (AP: -3.3, ML: ±2.0, DV: -2.8 to -3.3mm). Hippocampal traces were recording as the power of the theta oscillation is a sensitive index of the degree of forebrain activation. In some experiments additional bipolar electrodes staggered vertically by 1.5-2.00mm were implanted in frontal cortex (AP: +2.6mm, ML: ±0.5mm, DV(deep pole): -1.5-2.0mm) , (Contreras, Destexhe, Sejnowski, & Steriade, 1997; Molle, Yeshenko, Marshall, Sara, & Born, 2006; Siapas & Wilson, 1998). Indifferent (reference) electrodes (used when not

referencing signals to ground) were constructed from either an electrically connected pair of thick Teflon insulated wires (200μm: A-M Systems Inc. Carlsborg, WA.) staggered by 1.5mm or a single wire of the same type scraped bare to a distance of at least 1.5mm, implanted in the frontal hemisphere (AP: +1.0mm, ML: ±1.5mm, DV: -1.5mm). Indifferent electrodes were verified to be electrically neutral by comparison to ground (Wolansky, Clement, Peters, Palczak, & Dickson, 2006). Stimulation electrodes were constructed of twisted bipolar Teflon-coated stainless steel wires and were aimed at either the posterior hypothalamus (AP:-3.3mm, ML: ±0.1mm, DV: -8.5mm) or the pedunculo pontine tegmental nucleus (AP:-8.0mm, ML: ±1.8mm, DV: -7.8mm). Cannulae were constructed from blunt ended 30-gauge stainless steel hypodermic needles and were aimed at the basal forebrain region (AP:-0.5mm, ML: ±2.5mm, DV: -8.0mm). In some animals, needle electrodes were implanted bilaterally in the subcutaneous layer of the chest in order to monitor cardiac (EKG) activity. In these same animals, a thermocouple wire (30 gauge Type K; Thermo Electric Co., Inc.; Brampton, ON, Canada) was placed just inside the nasal passage to monitor respiration rate (Chaput & Holley, 1980). In other animals EOG was recorded with teflon-coated silver wire electrodes, implanted in to the external canthus muscle as described previously (Datta & Hobson, 2000). Following implantation, all electrodes and connecting wires (except for EKG and respiration leads) were fixed to the skull using jeweler's screws and dental acrylic.

Recording procedures. During recordings, the stereotaxic apparatus was connected to ground. Field potential and EOG recordings were amplified at a gain

of 1000 and filtered between 0.1 to 500Hz using a differential AC amplifier (Model 1700, A-M Systems Inc.). All field signals were referenced to the implanted indifferent electrode or to ground. Comparison of the two configurations ensured that no referencing artifacts ensued (Wolansky et al., 2006). EOG signals were referenced to each other. EKG leads were referenced to each other, amplified at a gain of 1000 and bandpass filtered between 10 to 500Hz. Thermocouple signals were amplified at a gain of 10000 and filtered between 0.1 and 500Hz. This method yielded a continuous representation of the respiratory cycle due to the temperature difference between inspired and expired air (Chaput & Holley, 1980). All recorded signals were digitized on-line (sampling frequency 1kHz) with a Digidata 1322A A-D board connected to a Pentium PC running the AxoScope acquisition program (Axon Instruments; Union City, CA).

Manipulations: Systemic pharmacological manipulations using atropine sulphate (50mg/kg), m-oxotremorine (11.17±3.96mg/kg) and physostigmine (2.39 ± 0.9mg/kg) were injected either intraperitoneally (i.p.) or i.v. via the jugular or femoral vein. Atropine sulphate is a muscarinic antagonist, while oxotremorine is a muscarinic agonist. Both these drugs induce their effects by actions at the receptor and confirmation of pharmacological effects was established by changes in the electrographic recordings. Physostigmine agonizes cholinergic neurotransmission by inhibiting cholinesterase, and thus inhibiting the breakdown of acetylcholine within the synapse. Confirmation of its pharmacological effect was established by visual examination of the animal for small peripheral twitches.

Pre-treatment using reserpine (5mg/kg) involved i.p. injections of the drug 14-18 hours prior to the beginning of the experiment. Confirmation of its pharmacological effect was established by gross behavioral observations subsequent to the administration in the home cage when the animal displayed behavioral depression characterized by sedation, ptosis and akinesia (Bueno, Pscheidt, & Himwich, 1968; Carlsson, Lindqvist, & Magnusson, 1957; Carvalho et al., 2006). Site specific inactivation of the basal forebrain was conducted by slowly infusing (1μl/min) lidocaine, which blocks sodium conductance, via a microinfusion pump (Model KDS100, KD Scientific Inc. Holliston, Massachusetts) through PE-50 tubing (Fisher Scientific, Ottawa, Ontario) attached to the implanted cannula. Stimulation of the posterior hypothalamus (PH) or pedunculo pontine tegmental nucleus (PPT) was conducted with biphasic square-wave pulses, 0.1msec in duration at a frequency of 100Hz for a duration of 5 to 15 seconds (Model 2100, A-M Systems Inc. Carlsborg, Washington). Current amplitudes used ranged from 50μA to 900μA. In all experiments, adequate baseline recordings (consisting of four complete alternations) were taken prior to manipulations.

Variation in depth of anesthesia across states was quantified using a nociceptive infrared source producing a heating beam placed beneath the tail or hind paw (Model 7371 Plantar Test, Ugo Basile; Biological Research Apparatus, Comerio, Italy). This device automatically detected withdrawal latency to the nearest 0.1s. Stimulations were administered across alternations in order to acquire latencies in both activated and deactivated states.

Chronic (freely-behaving) preparation.

Anesthesia and surgery. Animals were anesthetized with an i.p. injection of a ketamine/xylazine cocktail (90 and 10mg/kg, respectively, Steriade, Nunez, & Amzica, 1993). Rats were also administered a subcutaneous dose of atropine methyl nitrate (0.05 mg/kg) to prevent respiratory complications. During anesthesia, body temperature was maintained at 37°C and level of anesthesia was assessed as described previously. Supplements of the ketamine/xylazine cocktail (10% of original dose) were administered as necessary to maintain the animal's level of anesthesia.

Stereotaxic procedures. Using antiseptic techniques, animals were implanted stereotaxically (as described above) with unilateral frontal neocortical and bilateral hippocampal electrodes. A wire soldered to a skull screw placed over the cerebellum or an uninsulated electrode implanted vertically in the frontal nCTX served as a signal reference. All intracerebral electrodes were manufactured by Plastics One (Roanoke, VA). Bipolar electromyographic (EMG) electrodes (constructed from the same wire used for field recordings) were implanted in the neck musculature as described elsewhere (Whelan, 2003). All wires and connector assemblies were fixed to the skull using jeweller's screws and dental acrylic. Following implantations, the scalp was cleaned and sutured, and the animal was placed in a clean cage.

Recovery. Animals were allowed to recover for a minimum of one week prior to any recording. During this time they were handled on a daily basis and habituated to the recording apparatus for at least two hours each day during the

light cycle. During the habituation procedure, all leads were connected to suspended wires and animals were allowed to freely behave in the recording chamber which was contained in a Faraday cage and housed in a quiet room. Adequate habituation ensured that animals slept during recording sessions.

Recording procedures. Field signals were amplified and digitized as described above. Potentials from bipolar EMG electrodes were referenced to each other, amplified at a gain of 10000 and bandpass filtered between 10 to 500Hz. Multi-site field recordings were made simultaneously during ongoing (spontaneous) behavior for a variable time period (1 to 8 hours) daily. Periods of immobility were identified in electrographic recordings by low EMG tone and were classified as sleep only after offline confirmation using temporally correlated video records. Sleep and wake states were distinguished visually using both posture and open versus closed eyes. During sleep, REM and nREM episodes could be differentiated by the presence or absence of activated patterns of EEG in the nCTX (low voltage fast activity: LVFA) and HPC (theta), respectively. Further confirmation was obtained with EMG recordings; a decrease in EMG tone was concomitant with nREM to REM alternations.

Following the acquisition of a minimum of 5 complete REM/nREMrats were prepared for acute (urethane) anesthetized recordings as described in the previous section. Recordings during anesthesia were taken using the same connector pins used for naturally sleeping recordings and included EMG.

Perfusion and histology.

Following anesthetized recording sessions, small lesions were made at the tips of active intracerebral electrodes by passing 0.1 to 1mA of D.C. current for 5s using an isolated constant current pulse generator (Model 2100, A-M Systems Inc.). Rats were perfused transcardially, initially with physiological saline, and then with 4% paraformaldehyde in saline. Brains were extracted and stored overnight in 30% sucrose in 4% paraformaldehyde. The tissue was frozen with compressed CO_2 and sliced at 48μm with a rotary microtome (Leica 1320 Microtome; Vienna, Austria). Slices were then mounted on gel-coated slides, allowed to dry for a minimum of 24 hours, stained using cresyl violet or thionin, and coverslipped. Microscopic inspection of stained slices was used to verify recording, stimulating and infusion loci (Wolansky et al., 2006). Digital photomicrographs (Canon Powershot S45; Tokyo, Japan) were taken on a Leica DM LB2 (Buffalo, NY) microscope, imported using Canon Remote Capture 2.7 software (Tokyo, Japan) and processed with Corel PhotoPaint (Ottawa, ON, Canada).

Data processing and analysis.

Raw signals were first examined visually using AxoScope (Axon Instruments) and converted to ASCII format for further analysis. Zero-phase distortion digital filtering, single and dual-channel spectral analyses, and auto- and cross-correlations of field, respiration and heart rate activity were conducted offline using Matlab Version 5.1/5.3 (Mathworks; Natick, MA) and visualized using Origin (Microcal Software Inc.; Northampton, MA).

Autopower spectra were computed and plotted for field signals. Static spectra were computed from data segments whose length depended upon the type and stationarity of the signal. For segments of activated patterns with higher frequency components such as theta, segments were at least 30s. For deactivated patterns with lower frequency components such as LIA and slow oscillations, segments were at least 60s. Spectra were computed using a series of 6s long, Hanning-windowed samples with 2s overlap, across the entire data segment. Spectral values at peak signal frequencies or across a peak frequency bandwidth (comprising a range of 0.3Hz) were calculated and compared across EEG states. Spectrograms were computed from even longer data segments (several minutes) in which state alternations took place. A sliding windowing procedure was adopted that allowed discrete spectra to be calculated for specific time points across the entire data segment. Windows were 24s in duration and were moved across the data segment in increments of 6s. Spectral analysis of these individual samples was identical to the methods described above. Period analysis was performed by creating a sliding spectrogram of acquired data to visualize dynamics of all frequencies across time. The power at 1Hz was extracted from this data and plotted separately since it showed the highest fluctuation across state changes. This method facilitated the temporal (period) analysis of state changes.

To identify spindles, we used methods similar to previous investigators (Eschenko, Molle, Born, & Sara, 2006). In brief, I decimated data for a sampling frequency of 100Hz, and applied a band-pass filter of 7-10Hz to cortical traces. The root mean square (RMS) value was calculated at every time point using a

window of 0.2 seconds. Both the mean and standard deviation (SD) of RMS were calculated for individual files. A threshold value (mean +3 SD) was set to identify spindles. Duration of a spindle was acquired by calculating the time difference between when the RMS superseded a value of one SD greater than the mean prior to and after crossing the threshold value. Any spindles that had a duration of less than 0.5s were discarded. The temporal occurrence of spindles relative to the evolution of the activated-deactivated alternations was assessed by extracting the spectrographic power at 7, 8 or 9 Hz and comparing it to the 1 Hz spectrographic power (described above). The temporal relationship between the two time series power signals was explicitly examined using cross-correlation analysis. The data were summarized across experiments by standardizing the alternation periods as a complete cycle (i.e. from 0 – 360 degrees) and creating an average cross-correlation.

Heart and respiration rates were also quantified using spectrographic measures, with a frequency resolution of 0.05Hz over a period of 1/6s. Peak frequencies were then extracted and plotted across time and variations in these frequencies were compared across states and state alternations using chi^2 analysis.

EMG analysis consisted of peak-to-peak quantification across states. Normalization across animals, for the purpose of pair-wise comparisons, was done by representing the activated or REM state as a percentage of the value for the deactivated or nREM state. Percent declines in tone were then acquired by subtracting the percent value of the activated or REM state from 100.

Data designed to assess behavioural level of anesthesia was organized such that stimulations given within the occurrence of a state were grouped and termed as a set. Changes in the sensitivity of the behavioural response as a function of stimulus presentation were established by performing linear regression analysis on the raw data of two temporally adjacent sets. If significance was found ($p<0.05$) within two consecutive linear analyses (activated-deactivated and deactivated-activated) then the common set was discarded. This occurred only once. Significance tests were performed similarly, using pair-wise t-tests of average withdrawal latencies of adjacent sets within an animal.

Summary reports of data across experiments or conditions in the Results section are reported as arithmetic means together with the standard error of the mean (SEM) unless otherwise noted. Numerical comparisons across conditions for the same datasets were made using two-tailed pair-wise t-tests (with a significance level of 0.05). As most tests were specified a priori, this was also the case for datasets with more than two conditions. For remaining situations, data were analyzed with t-test assuming either equal or unequal variance (depending on whether variance, represented by the coefficient of variance, was significantly different across data sets) or one-way ANOVA (using the Tukey post-hoc corrected tests) analyses with significance at a level of 0.05.

Drugs and chemicals.

Atropine methyl nitrate, atropine sulphate, reserpine, physostigmine, lidocaine, m-oxotremorine, ethyl carbamate (urethane), and thionin were all purchased from Sigma (St. Louis, MO). All reagents were mixed in double

distilled water. Reserpine solutions were made by first dissolving 10mg in 50uL glacial acetic acid, then adding double distilled water to make a final solution of 1ml. Isoflurane and ketamine were purchased from Bimeda-MTC (Animal Health Inc.; Cambridge, ON, Can). Cresyl violet was purchased from Acros Organics (Morris Plains, NJ), paraformaldehyde from Fisher Scientific (Toronto, ON, Can), and xylazine from Bayer Inc. (Toronto, ON, Can).

Results

Spontaneous and rhythmic brain state alternations under urethane anesthesia.

In long term (>45 minute) field (EEG) recordings made from neocortical and hippocampal sites in urethane-anesthetized rats, a spontaneous and highly regular alternation of electrographic state between activated and deactivated patterns was noted. Activated EEG patterns consisted of low amplitude fast activity at neocortical sites concomitant with theta (rhythmic 3-5 Hz) activity at hippocampal sites while deactivated EEG patterns were characterized by large-amplitude slow oscillatory (~1Hz) activity at both neocortical and hippocampal sites (Fig. 1A). As previously reported (Wolansky et al., 2006), the spontaneous evolution between these states was stereotyped and involved a gradual transition from the activated to the deactivated state (5.23 ± 0.60 minutes, range: 2.75 to 8.58, n=10) and then a rapid (52 ± 4s, range: 34 to 78, n=10) shift back into the activated state (Fig. 1B). This cycle repeated itself in a stable and consistent rhythmic fashion as demonstrated by observation of the amplitude fluctuations of long-duration plots of raw EEG traces (Fig. 1C) and spectrographic plots

Figure 1. **Urethane anesthetized animals displayed spontaneous and cyclic alternations of brain state.**

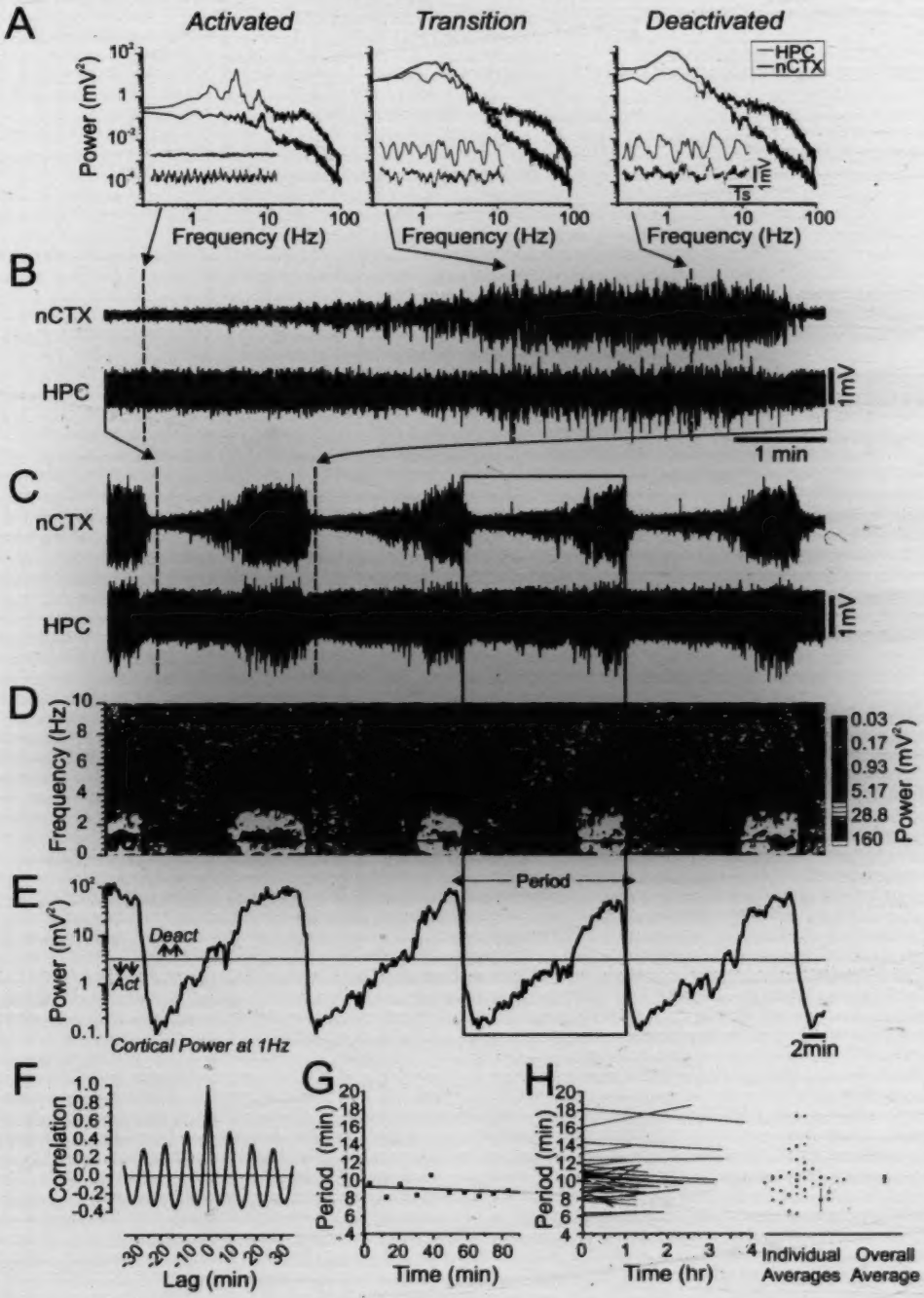

Figure 1. Urethane anesthetized animals displayed spontaneous and cyclic alternations of brain state.

A) Spectra and raw EEG traces of different spontaneous states under urethane. During the activated state, the nCTX showed low voltage fast activity and the HPC showed prominent theta at ~4Hz. During the transition state, there was an increase in overall power with a shift to lower frequencies at both sites; raw EEG traces showed irregular and moderately high amplitude activity. During the deactivated state, there was an even more prominent shift to low (~1Hz) frequencies with a further increase in overall power and raw EEG traces displaying prominent rhythmic high amplitude slow activity. **B)** Continuous EEG traces of a spontaneous transition from activated to deactivated patterns. Positions from where the expanded traces in A were taken are shown. The transition between activated and deactivated patterns was marked by a gradual increase in the amplitude of the signals whereas the transition between deactivated and activated patterns was more abrupt. **C),** Continuous EEG traces over an even longer time scale demonstrated a regular and cyclic alternation of state as observed by the fluctuations in amplitude. The position from where traces in B were taken are shown. **D)** A spectrographic representation of the neocortical trace shown in C. The most prominent fluctuation of power was centered at a frequency of circa 1Hz. **E)** Plot of power at 1 Hz from the spectrograph in D. The power values showed a cyclic fluctuation in amplitude continuing over the entire time of recording with an average period of ~9 minutes. **F)** Autocorrelation of power values from the experiment shown in E which showed a prominent rhythmic fluctuation at a similar period (9 min). **G)** Scatter plot of alternation period across time. The period length for the experiment illustrated remained stable as shown by the linear fit with a slope value not significantly different from zero ($p=0.46$). **H)** The left panel shows regression lines for cycle periods across time for all experiments having 6 or more cycles. Different experiments are represented by different colored lines and demonstrate a general lack of variation within, but some variation across, animals. Thirty eight of the forty one animals displayed here showed no significant regression at a p level of 0.05. The right panel is a scatter plot of the average periods for each experiment (equivalent colors to the right panel) and the overall average (10.3 ± 0.4 min) across all experiments.

computed on the same EEG traces (Fig. 1D). Within spectrograms, the highest power fluctuations were centered at a frequency of 1Hz (especially in the neocortex) so the period and rhythmicity of the alternations were systematically characterized by extracting this band across time (Fig. 1E). The precise time points of alternations between states were denoted by calculating a threshold value (corresponding to the saddle of the bimodal distribution of the power at 1Hz across time) which separated activated from deactivated patterns (values below the threshold were considered to be activated while those above were considered to be deactivated). Autocorrelations of the detrended power at 1Hz were highly rhythmic (Fig. 1F) and successive periods maintained a consistent duration across time (Fig. 1G), both consistent with a systematic cyclic process. Least-squares linear fitting of these durations across time provided regression coefficients (slopes) that were not significantly different from zero (i.e. showed no systematic linear relationship with time) in 38 out of 41 experiments (Fig. 1H).

These rhythmic state alternations were a consistent finding in 79 out of 83 animals and had an average period of 10.5 ± 0.3 minutes. Of the four animals that did not show this effect, one showed a consistent deactivated electrographic pattern and the other three showed irregular (arrhythmic) and inconsistent alternations between activated and deactivated patterns.

Although previous investigators have noted spontaneous state changes under urethane anesthesia, none have described the consistent, rhythmic and systematic alternations reported above (*cf.* (Ylinen et al., 1995)). State changes under urethane have typically been ascribed to variations in anesthetic level (Detari,

Semba, & Rasmusson, 1997; Grahn, Radeke, & Heller, 1989; Murakami, Kashiwadani, Kirino, & Mori, 2005). However, this interpretation is inconsistent with prior metabolic studies of urethane, and with my own observations of 1) the effects of supplemental doses of urethane, and 2) the behavioral assessment of anesthetic level. Although urethane is rapidly and evenly distributed throughout bodily tissues, it is metabolized very slowly in rodents and shows a constant (i.e. non-fluctuating) rate of decline over time as measured in the blood (Nomeir, Ioannou, Sanders, & Matthews, 1989; Sotomayor & Collins, 1990). Moreover, supplemental doses of urethane (ranging from 0.07 to 0.27mg/kg), while decreasing the relative amount of time spent in the activated state ($8.9 \pm 2\%$ for every 0.1mg/kg administered, n=5), did not alter either the presence or the rhythmicity of state alternations per se (Fig. 2). No significant differences were observed for the measure of alternation periods pre- and post-supplement in any individual experiment ($p \geq 0.15$) nor overall ($p=0.68$). Finally, a behavioral assay of anesthetic level was found to be consistent across states. In initial experiments, I assessed withdrawal to sharp and consistent pressure applied on the hind paw pad within each state. Although I could find no differences using this technique I further (and more systematically) examined level of anesthesia by measuring withdrawal using a ramped heat tail-flick apparatus (see Methods section). Although the intensity of the infrared beam had to be maximized in order to elicit even minimal withdrawal (consistent with a surgical plane of anesthesia) a pair-wise t-test performed on temporally adjacent sets (average of latencies within the

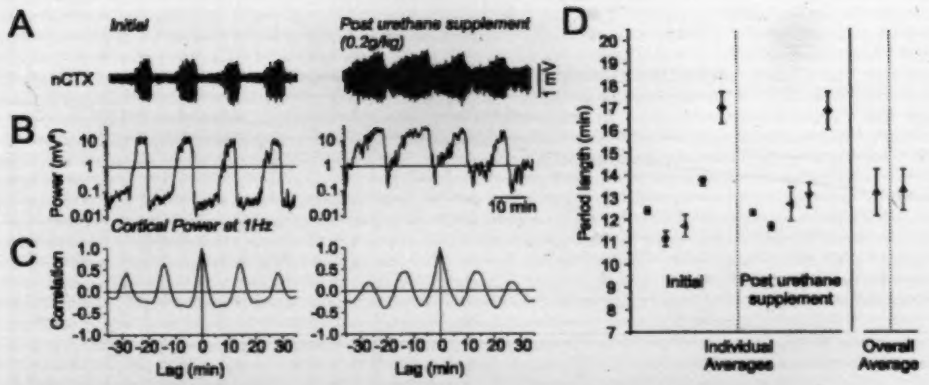

Figure 2. The rhythmicity and periodicity of state alternations under urethane were not affected by moderate increases in anesthetic dosage.

A) Long duration raw cortical EEG traces demonstrating the electrographic effects of a moderate increase in the depth of urethane anesthesia. Although the overall amplitude of the signal increased following the supplemental dose, state alternations (apparent as rhythmic changes in signal amplitude) were still observed. **B)** Spectrographic power at 1 Hz for the traces shown in A. Following the supplemental dose, power continued to fluctuate at a similar periodicity although there was an increase in the overall power in addition to the time spent in the deactivated state (and consequently a decrease in the time spent in the activated state). **C)** Autocorrelation of power values in B demonstrating similar rhythmicity before and after supplemental urethane administration. **D)** Individual (paired for animal across conditions by symbol) and overall averages demonstrating the consistency of alternation period duration before and after the supplemental doses of urethane across all experiments. Neither the individual nor the overall averages were significantly different.

occurrence of a state) revealed no significance difference in withdrawal latency between activated and deactivated states (Fig. 3, n=10, p=0.31).

State alternations under urethane anesthesia resemble natural sleep state alternations.

Brain state fluctuations are a well known phenomenon during natural sleep. Prior measurements of the duration of REM/nREM sleep cycles in rats have a distribution with a mean and modal value between 9.5 and 13.5 minutes (Borbely, 1976). This timing overlaps extremely well with the distribution that I found for state alternations under urethane. To directly evaluate the similarity of the two conditions, I performed recordings in naturally sleeping rats that were later anesthetized with urethane (Fig. 4). The raw electrographic characteristics of REM sleep were highly similar to those demonstrated in the activated state under urethane anesthesia, with the cortex displaying low-voltage fast activity (LVFA) and the hippocampus eliciting a prominent theta rhythm. The spectral characteristics of both states, assessed by comparison of peak frequencies and power, were likewise similar although the peak frequency of theta was significantly higher on average in REM (6.4 ± 1.2Hz) than in the activated state during urethane (4.1 ± 0.1Hz, p=0.001) (Fig. 4A, B, C). Likewise, during slow-wave sleep and the deactivated state under urethane anesthesia, both cortical and hippocampal traces were dominated by the slow oscillation (Wolansky et al., 2006) and spectra showed overlapping distributions without significant differences in either peak frequency or power (Fig. 4A, B, C).

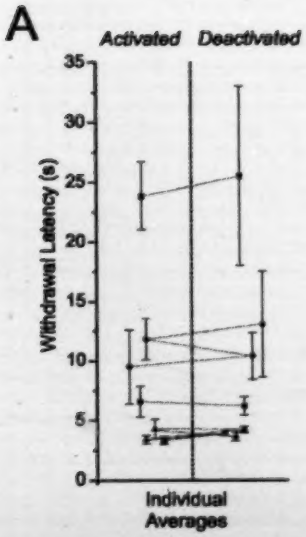

Figure 3. Anesthetic level was comparable across state alternations.

A) Averages of withdrawal latencies in response to a ramped infrared beam applied to the hindpaw. Stimulations given within the occurrence of a state were grouped into sets and represented by one average on the plot. A pair-wise t-test, (pairs are connected on the plot by lines) on temporally adjacent averages revealed no significant difference in withdrawal latency across activated and deactivated states (n=10, p=0.31). Animals are coded by symbol.

Figure 4. State-dependent characteristics of forebrain EEG were similar across natural sleep and urethane anesthesia.

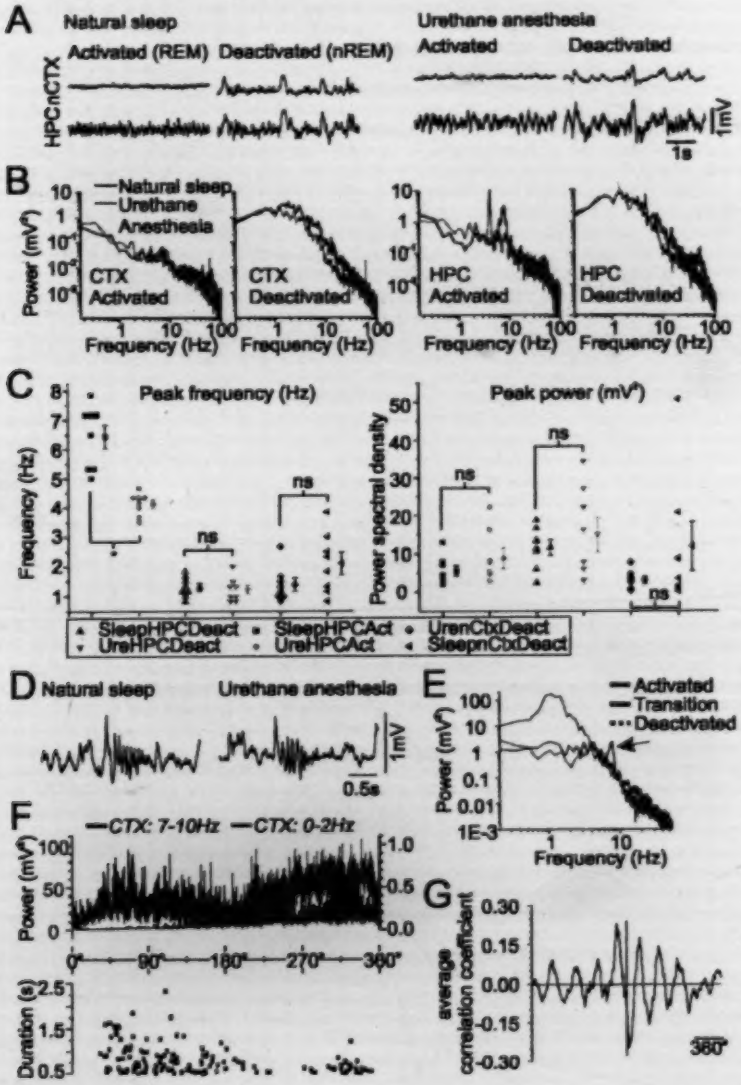

Figure 4. State-dependent characteristics of forebrain EEG were similar across natural sleep and urethane anesthesia.

A) Expanded neocortical and hippocampal EEG traces across natural sleep and urethane anesthesia in the same animal showing examples of activated and deactivated patterns in both situations. Regardless of condition, activated and deactivated patterns were highly similar. **B)** Spectra of cortical and hippocampal EEG traces overlaid across conditions for the same electrographic patterns. Although the peak frequency of hippocampal theta power during REM was at a higher frequency (~7Hz) than during the activated state under urethane anesthesia (~4Hz), all other spectra across conditions appear highly similar. **C)** Scatter plot of peak frequencies (left panel) and power (right panel) for hippocampal signals during the activated state and neocortical and hippocampal signals during the deactivated state for each animal. Except for the peak frequency of hippocampal signals during activated patterns, there were no significant differences across natural sleep and urethane anesthesia. **D)** An expanded example of a cortical spindle oscillation recorded during natural sleep (left panel) and a similar pattern recorded at the same site the transition phase under urethane anesthesia (right panel). **E)** Superimposed spectral plots of cortical EEG taken during activated, transition and deactivated states in a urethane-anesthetized animal. Note the spectral peak in the transition spectra centered at ~8Hz (spindle frequency). **F)** Spectrographic power from 0 to 2Hz (grey) and 7 to 10Hz (black), and the occurrence and duration of spindles across the evolution of all state alternations for one full experiment. Note the enhanced presence of spindling activity at the transition points between activated and deactivated patterns. **G)** Average (across all experiments) of the cross correlation function between 1Hz and spindle (7-9Hz) spectrographic power (n=5; standardized cycle in degrees). The temporal relationship of spindling to slow oscillatory activity at 1Hz patterns showed a consistent lag of approximately half a period length (~180 degrees).

In addition, another electrographic similarity was apparent during the transition period between activated and deactivated patterns in both conditions. During this period, the cortical EEG traces showed spindle activity, often with a characteristic K-complex-like spike followed by an envelope of high frequency (7-15Hz) oscillations lasting longer than 0.5s. As shown in Figure 4D, these events were remarkably similar across both natural sleep and urethane anesthesia as recorded in the same animals. In fact, during urethane anesthesia, spindle frequencies were a consistent facet of the transition period as demonstrated by a spectral peak in the 7-10Hz range (Fig. 4E). In long-duration recordings under urethane, spindle events were more probable and of higher duration in the intervening period between activated and deactivated states (Fig. 4F). As well, increases in spindle frequency power (bandwidth 7-10Hz, average: 8.2 ± 0.4Hz, n=5) consistently led increases in 1Hz (i.e. slow oscillation) power at an average lag of 170 degrees (i.e. at approximately the half-cycle point) with respect to the rhythmic activated-deactivated state alternation (Fig. 4F,G).

Not only were the electrographic aspects of individual states similar across natural sleep and urethane anesthesia, but the average alternation period between these states was also highly comparable (Fig. 5). The same animals recorded across conditions showed an average cycle length of 11.1 ± 0.5 minutes in natural sleep and 11.3 ± 0.6 minutes when later recorded under urethane anesthesia (Fig. 5B). This difference was not significant (n=8, two-tailed pair-wise t-test, p=0.73). Not surprisingly, however, as responses to sensory stimuli are maintained in natural sleep whereas they are absent in anesthesia, there was significantly greater

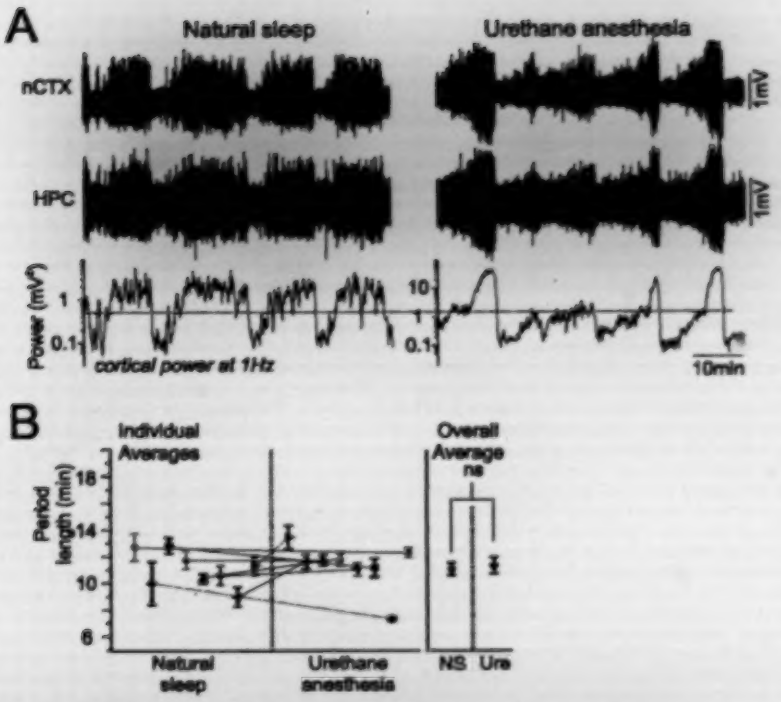

Figure 5. The timing of cyclic EEG state alternations was highly similar across naturally sleeping and urethane-anesthetized conditions.

A) Long-duration EEG traces during a continuous natural sleep episode (left panel) and subsequently from the same animal under urethane anesthesia (right panel). Regular fluctuations between REM and nREM sleep stages were highly comparable in their timing to the state alternations later observed under urethane. Plotted on the same time scale underneath the raw traces is the respective spectrographic power at 1Hz extracted from the cortical signals. These fluctuations demonstrated a similar rhythmicity across both conditions. **B)** Scatterplot representing period lengths of alternations for all animals under both naturally sleeping and urethane-anesthetized conditions. Solid lines represent comparisons across conditions that were not significantly different (n=6). Perforated lines represent those comparisons that demonstrated significance across conditions (n=2). Despite this, similarity in average length of alternations across conditions is substantiated by a lack of significance when comparing overall averages in a pair-wise manner across both naturally sleeping and urethane anesthetized conditions.

variance in cycle length across conditions (19.3 ± 2.3s in natural sleep versus 7.2 ± 0.6s in urethane anesthesia, n=8, pair-wise t-test, p=0.001).

These data suggest a similarity in the brain mechanisms responsible for both the induction and maintenance of unconsciousness across both sleep and urethane anesthesia. To assess the similarities of other physiological processes know to covary with sleep states I evaluated eye movements (EOG), muscle tone (EMG), heart rate (EKG) and respiration rate (Aserinsky & Kleitman, 1953; Aserinsky, 1965; Dement & Kleitman, 1957; Pace-Schott & Hobson, 2002; Sinton & McCarley, 2004) during EEG state alternations under urethane anesthesia.

EOG signals were non-existent under urethane anesthesia and showed no differential activity across states (n=3). Indeed, in contrast to sleep, rats under urethane typically maintained open eyelids.

My EMG recordings during urethane, however, did show changes consistent with natural sleep. Despite the fact that urethane induces muscular atonia itself (Robinson, Kramis, & Vanderwolf, 1977) (and supported by significantly lower average peak-to-peak [RMS] values of EMG during urethane [1.36 ± 0.55mV] as compared to sleep [2.67 ± 1.06mV: one-tailed pair-wise t-test, p=0.04, n=5]) in 3 of the 5 animals which had functional EMG recordings during natural sleep, I observed similar and significant (all pair-wise comparisons significant at $p<0.01$) decreases in EMG tone from deactivated to activated states during subsequent recordings during urethane (average decline across deactivated to activated states of 21.2 ± 6.1%, during sleep as compared to 11.8 ± 3.0% under urethane) (Fig. 6A, B). Consistent with a depression of EMG tone generally, this decline under

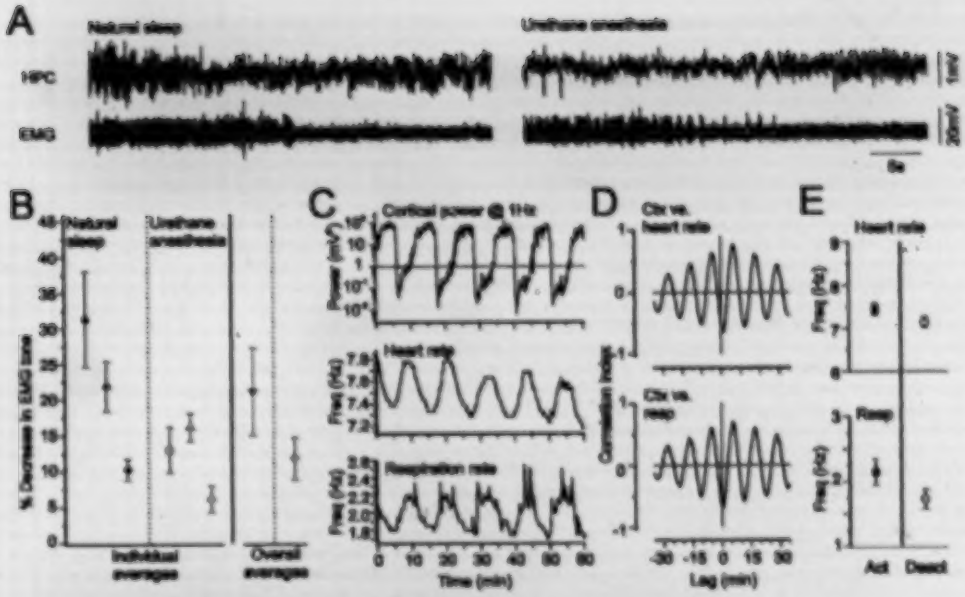

Figure 6. Physiological correlates of EEG state changes were similar across naturally sleeping and urethane-anesthetized conditions.

A) Simultaneous recordings of hippocampal field activity and neck EMG in naturally sleeping (left panel) and urethane anesthetized (right panel) conditions in the same animal. Declines in EMG tone with transitions from nREM to REM sleep were also apparent under urethane anesthesia with transitions from deactivated to activated EEG patterns. **B)** In 3 animals, the average percentage loss of EMG amplitude across these transitions was consistent and significantly different across both conditions, although it was smaller due to the general depression of EMG tone under urethane. **C)** Simultaneous extraction of cortical spectrographic power at 1Hz (top panel), heart rate (middle panel) and respiration rate (lower panel) demonstrating concomitant fluctuations. Increases in both heart and respiration rates appeared during the lowest EEG power readings (i.e. the activated state). As well, during the activated state, the respiratory cycle tended to show greater variation. **D)** Fluctuations in heart (top) and respiration rates (bottom) were rhythmically correlated with state changes as shown in the cross correlation of these variables to cortical power at 1Hz. **E)** Summary data across experiments showing significant increases in both heart (top) and respiration rates (bottom) when comparing the activated to the deactivated state.

urethane was smaller than that in natural sleep although the difference between conditions did not achieve significance ($p=0.09$). In the remaining two animals, there was no change in the EMG tone under urethane but this failure may have reflected a floor effect due to the general depression of muscular tone.

Lastly, and again similar to natural sleep, consistent and significant increases in both heart (0.29 ± 0.06Hz: 4.1 ± 0.8%) and respiration (0.42 ± 0.08Hz: 24.1 ± 4.1%) rates were concomitant with transitions from deactivated to activated states under urethane anesthesia (Fig. 6C). Furthermore, in 3 of 4 animals, respiration rate became significantly more irregular as characterized by higher variability (more frequent changes) during activated states (chi^2 analysis, $p<0.05$, $n=3$ and $p=0.90$, $n=1$). Thus, alternations in brain state exhibited under urethane anesthesia are correlated with physiological changes consistent with those exhibited during natural sleep.

State alternations under urethane anesthesia depend on brain systems known to influence natural sleep state alternations.

State alternations during sleep are known to be due to forebrain fluctuations in the release of endogenous acetylcholine (ACh) emanating from differential activity in ascending brainstem arousal systems (reviewed in (Hobson & Pace-Schott, 2002; Jones, 1993; Pace-Schott & Hobson, 2002; Steriade & McCarley, 1990; Steriade, 1999). I sought to test the cholinergic dependence of state alternations under urethane by: 1) demonstrating and contrasting the pharmacological effects of interfering with central cholinergic versus monoaminergic neurotransmission, 2) demonstrating the effects of temporary

inactivation of the major cholinergic afferent of the neocortex and hippocampus, the basal forebrain region, and 3) demonstrating the effects of electrical stimulation of ascending cholinergic brainstem nuclei such as the pedunculo pontine tegmental nucleus (PPT) and lateral dorsal tegmental nucleus (LDT) that are proposed to be elements of the pacemaking circuit for brain state alternations in sleep.

Systemic manipulations using the acetylcholinesterase inhibitor physostigmine (2.39 ± 0.9mg/kg, n=16) promoted a long lasting activated forebrain state (36.3 ± 6.2 minutes, Fig. 7 A, C) similar to the spontaneous activated state (Fig. 7B, Appendix A). Treatments using the muscarinic receptor agonist oxotremorine (11.17 ± 3.96mg/kg, n=11) also elicited activated forebrain EEG patterns similar to spontaneous activated patterns Fig. 8A, B, Appendix B) and this effect had a longer duration (70.1 ± 13.2 minutes, Fig. 8A, C). Treatments with the muscarinic antagonist atropine sulphate (50mg/kg, n=17) produced a deactivated state similar in spectral characteristics to spontaneous deactivated patterns (Fig. 7B and. 8B, Appendices A and B). This effect appeared non-reversible since no washout occurred over recording times as long as 100 minutes (average 46.9 ± 6.9 minutes). All of the above manipulations resulted in the abolition of state alternations, although as physostigmine was metabolized, a return to alternations into the deactivated state began taking place (Fig. 7A). These results demonstrate that spontaneous alternations of state in the urethane-anesthetized animal, like natural sleep, are also dependent on muscarinic mechanisms.

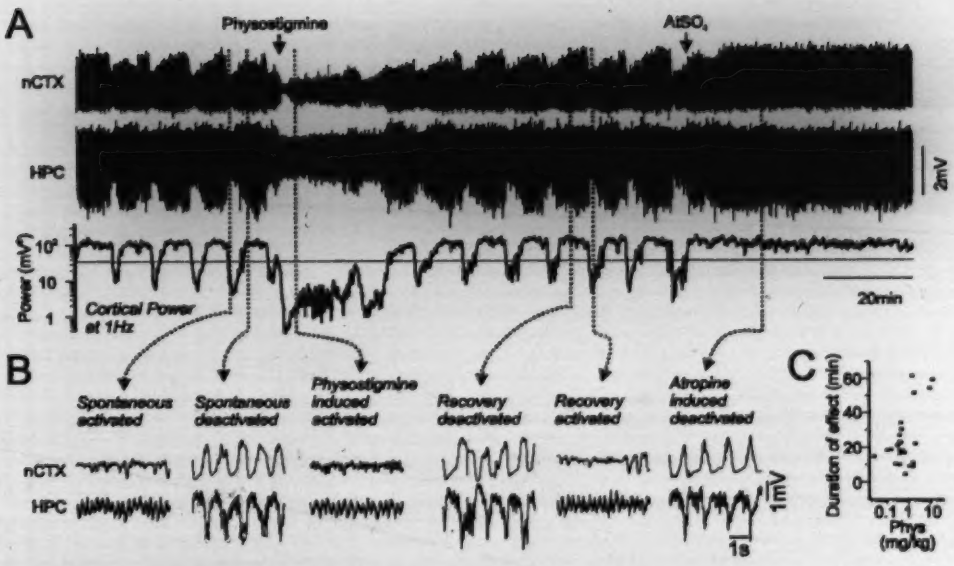

Figure 7. Cholinergic dependence of state alternations: Effects of physostigmine and atropine.

A) Ultra long duration cortical and hippocampal EEG traces in addition to spectrographic cortical power at 1 Hz demonstrating the effects of agonism and subsequent antagonism of cholinergic transmission. Following an i.v. injection of physostigmine (3.7mg/kg) spontaneous alternations between activated and deactivated states were temporarily abolished in favor of the activated state. Following recovery, state alternations were permanently abolished in favor of the deactivated state with a subsequent i.p. injection of atropine sulfate ($ATSO_4$: 50 mg/kg). **B)** Expansions of EEG traces from neocortical and hippocampal sites show the similarity of activated and deactivated patterns induced by cholinergic agonism and antagonism, respectively. **C)** Scatter plots demonstrating the duration of effects of physostigmine and atropine as a function of dosage. The effect of atropine never washed out even following lengthy subsequent recordings.

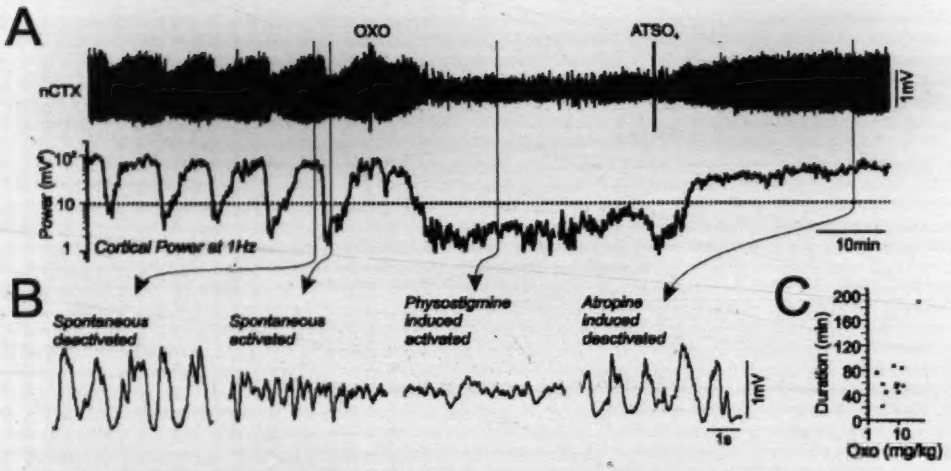

Figure 8. Cholinergic dependence of state alternations: Effects of oxotremorine and atropine.

A) Long duration cortical EEG traces in addition to spectrographic cortical power at 1 Hz demonstrating the effects of agonism and subsequent antagonism of muscarinic receptor mediated transmission. Following an i.p. injection of oxotremorine (4.0mg/kg) spontaneous alternations between activated and deactivated states were abolished in favor of the activated state. This induced activated state was abolished in favor of the deactivated state with a subsequent i.p. injection of atropine sulfate ($ATSO_4$: 50 mg/kg). **B)** Expansions of EEG traces from neocortical sites show the similarity of activated and deactivated patterns induced by cholinergic agonism and antagonism, respectively. **C)** A scatter plots demonstrating the duration of the oxotremorine effect as a function of dosage. The effects of oxotremorine were longer than those of physostigmine (Figure 6C). As in Figure 6, the effects of atropine showed no reversal.

A major difference between forebrain activation during wakefulness and during REM sleep is the role of ascending monoaminergic systems, most notably the noradrenergic system (Jones, 2003; Vanderwolf, 1988). In order to eliminate any possible contribution of these non-cholinergic systems in the elicitation of activated states and state alternations under urethane, I depleted monoaminergic vesicular stores by pre-treating animals with reserpine (5mg/kg) 14-18 hours prior to my experiments. The pharmacological action of reserpine was confirmed by behavioral observations in the intervening period for sedation, ptosis and akinesia (Bueno et al., 1968; Carlsson et al., 1957; Carvalho et al., 2006). Despite successful pre-treatments with reserpine, all subsequently anesthetized animals displayed spontaneous and rhythmic alternations of brain state demonstrating both activated and deactivated patterns (Fig. 9). Furthermore, a supplemental dose of reserpine (5mg/kg) during anesthesia affected neither the occurrence nor characteristics of the activated state nor did it affect the ongoing state alternations. Measurements were limited to hippocampal traces as variations in theta frequency and power are a sensitive index of the level of forebrain activation (Bland and Colom, 1993). As shown (Fig. 9B, C, D), raw traces and spectral components showed no differences pre- and post-reserpine supplement ($p \geq 0.926$ for both power and frequency measures). As well, the period length prior to supplement (8.16 ± 1.04 minutes) was not significantly different from that measured post-supplement (8.23 ± 1.04 minutes: $p=0.44$, $n=3$) (Fig. 9E). Therefore, individual states and their alternations under urethane anesthesia were not dependent upon the integrity of monoaminergic transmission as they are during the waking state.

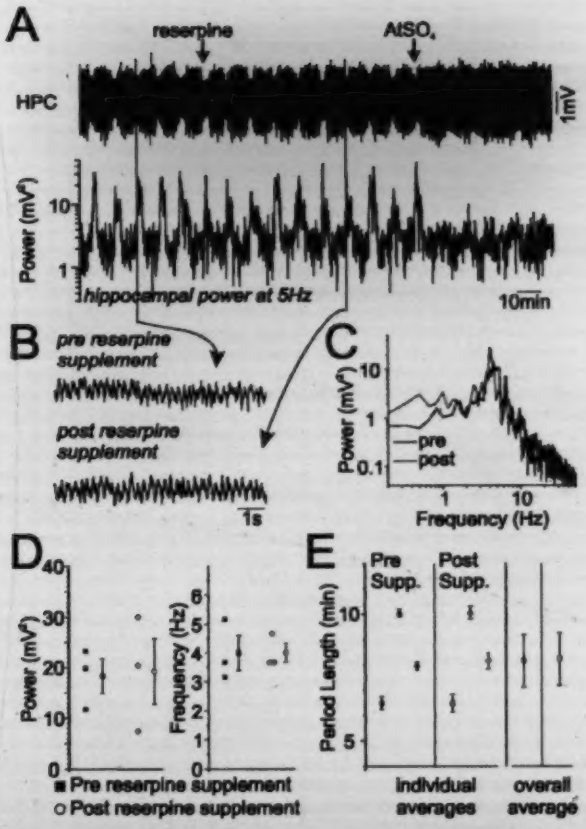

Figure 9. Monoaminergic depletion affected neither the activated state nor the alternations between states under urethane anesthesia.

A) Continuous hippocampal EEG traces and spectrographic power at theta frequencies under urethane anesthesia following reserpine (5 mg/kg) pretreatment. Alternations between states were obvious as fluctuations in the amplitude of the raw EEG in addition to the spectrographic theta power (5Hz). A further supplemental i.p. administration of reserpine (5mg/kg) was without effect upon either the activated state or the alternations between states. However, a subsequent i.p. injection of atropine ($ATSO_4$: 50mg/kg) completely abolished the activated state and subsequent alternations between state. Hippocampal **B)** EEG expansions and **C)** spectra from before and after reserpine supplement demonstrating intact theta activity in both cases. **D)** Scatterplots of peak power and frequencies for activated states pre and post reserpine supplement. There were no significant differences between pre and post supplement groups. **E)** Scatterplots of period lengths pre and post reserpine supplements demonstrating that alternations and rhythmicity were not affected by reserpine.

Since central cholinergic mechanisms appeared to be a major factor in controlling state alternations I next sought to investigate the role of structures in the brain that influence forebrain levels of acetylcholine. The direct source of ACh to the neocortex and the hippocampus emanates from nuclei in the BF which have also been implicated in the elicitation of activated patterns of the EEG during REM sleep (Jones, 2003). I assessed the role of these nuclei by temporarily inactivating these sites using direct intracerebral infusions of 1 - 4% solutions of lidocaine (22.5 ± 4.5μg: n=8). Infusions centered at sites such as the magnocellular preoptic nucleus, the substantia innominata, the horizontal limb of the diagonal band, the basal nucleus, and the ventral aspect of the globus pallidus (for a representation of the actual histological sites see Fig. 10D) produced a rapid (61 ± 10s) onset of a continuous deactivated pattern in the EEG of both the nCTX and HPC which lasted for an average duration of 26.1 ± 7.4min and resembled patterns elicited during spontaneous deactivation (Fig. 10A, C, Appendix C). During this time, no alternations of state took place. Following washout, state alternations returned. Additional equivolume infusions of lidocaine subsequent to washout at sites promoting deactivated states produced identical effects to the initial infusion (n=2) whereas increased volumes of infusion (n=5) resulted in an increase of time spent in the deactivated state. In cases in which infusion sites were outside of the BF (placements too lateral or ventral), forebrain EEG was not altered (n=2, for histological sites see Fig. 10D). Thus, as in sleep, the activity of specific nuclei within the BF region appears to be necessary for the expression of activated states and for the alternation of states under urethane anesthesia.

Figure 10. Local pharmacological inactivation of the basal forebrain region reversibly induces a deactivated state and temporarily abolishes state alternations.

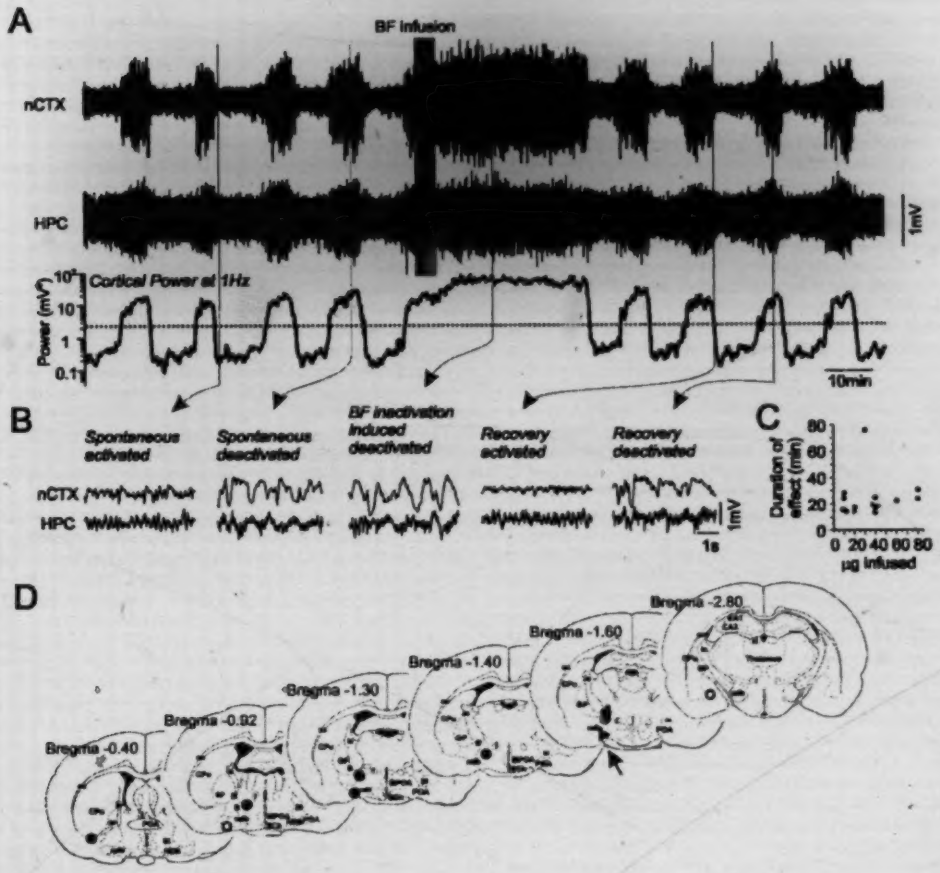

Figure 10. Local pharmacological inactivation of the basal forebrain region reversibly induces a deactivated state and temporarily abolishes state alternations.

A) Continuous long duration cortical and hippocampal EEG traces together with spectrographic cortical power at 1 Hz during an infusion of lidocaine in the basal forebrain (BF). Spontaneous state alternations were temporarily abolished in favor of a continuous activated state following inactivation of the BF. **B)** Expanded EEG traces from neocortical and hippocampal sites prior and following the infusion. Deactivated patterns were elicited during inactivation of the BF and these effects washed out with time. **C)** Duration of evoked deactivated activity as a function of the amount of lidocaine infused in the BF. **D)**. Histological representation of infusion sites. White circles indicate ineffective sites. Site marked with arrow and triangle denotes experiment shown in A and B. Abbreviations: B: basal nucleus, CPu: caudate putamen, ec: external capsule, f: fornix, GP: globus pallidus, HDB: horizontal limb of the diagonal band of Broca, ic: internal capsule, mfb: medial forebrain bundle, MPOA: medial preoptic area, POA: preoptic area, SCh: suprachiasmatic nucleus, SI: substantia innominata.

Although the basal forebrain (BF) is the direct source of forebrain acetylcholine, it is the cholinergic nuclei in the brainstem, such as the pedunculo pontine tegmental nucleus (PPT) and lateral dorsal tegmental nucleus (LDT), together with other nearby structures, such as the lateral pontine tegmentum (LPT), venterolateral periacquaductal gray (vlPAG), sublaterodorsal nucleus (SLD) and precoeruleus (PC), which are thought to constitute the essential pacemaker for sleep state alternations themselves (Hobson, McCarley, & Wyzinski, 1975; Lu, Sherman, Devor, & Saper, 2006; McCarley & Hobson, 1975; Pace-Schott & Hobson, 2002; Steriade & McCarley, 1990). Therefore, I electrically stimulated these sites in the brainstem in an attempt to disrupt the ongoing state alternations under urethane.

As previously reported (Robinson & Vanderwolf, 1978; Vertes, 1982), stimulation trains delivered to sites in or near the PPT resulted in the immediate elicitation of activated patterns in forebrain EEG (Fig. 11A, B, Appendix D). As noted by these researchers, an increase in stimulation intensity resulted in a linear increase in the degree of forebrain activation as indexed by the peak frequency of hippocampal theta activity (Fig. 11C). More importantly, however, directly following even a short series of stimulation trains, there was a consistent and long-lasting deactivation of forebrain state (33.7 ± 5.0 minutes, n=4) during which time no spontaneous alternations occurred (Fig. 11A, D). Thus, stimulation of forebrain-activating brainstem sites implicated in the pacing of cyclical brain state alternations during sleep subsequently disrupts state cycling under urethane anesthesia. This effect was unlikely to have been mediated by stimulation-induced

Figure 11. Stimulation trains applied to the pedunculo-pontine tegmentum temporarily abolished alternations of forebrain state.

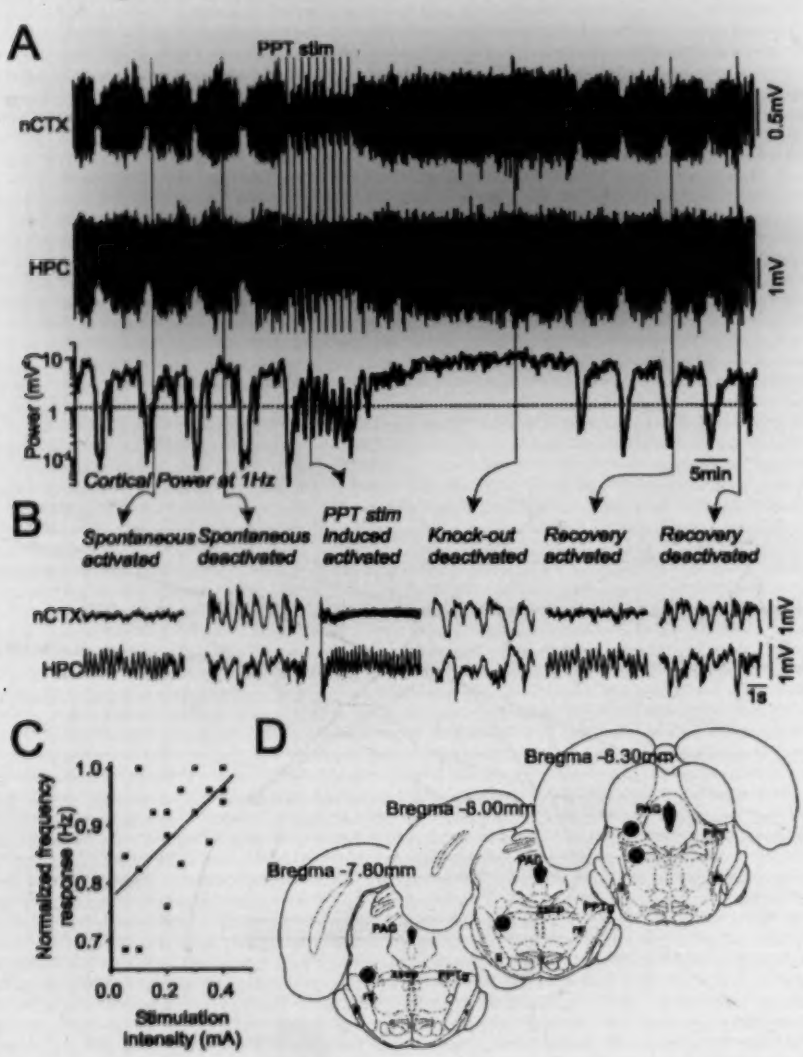

Figure 11. Stimulation trains applied to the pedunculo-pontine tegmentum temporarily abolished alternations of forebrain state.

A) Continuous cortical and hippocampal EEG traces and the spectrographic cortical power at 1 Hz demonstrating the effects of stimulation of the pedunculo-pontine tegmental (PPT) region. Subsequent to a series of moderate stimulation trains, which were each effective in promoting an activated forebrain EEG during application, there was a transient suppression of forebrain state alternations. **B)** Expanded EEG traces from neocortical and hippocampal sites demonstrate that activated patterns were elicited via stimulation and that deactivated patterns follow a stimulation train. **C)** Scatterplot and linear fit of frequency as a function of the stimulation intensity in the PPT showing a significant ($p<0.01$) relationship between stimulation intensity and the peak frequency of theta activity recorded in the hippocampus. The frequency was normalized across experiments to the maximal frequency of theta elicited in each. **D)** Summary of histological findings for the site of stimulation across experiments. Abbreviations: ll: lateral lemniscus, PAG: periaqueductal gray, PPT: pedunculo pontine tegmental nucleus, rs: rubrospinal tract, xscp: decussation of the superior cerebellar peduncle.

damage to brain stem sites responsible for forebrain activation since this disruption was only temporary. These effects were also unlikely to have been mediated by prolonged activation of forebrain regions per se since prolonged stimulation of posterior hypothalamic sites, which also potently activated forebrain EEG in a similar way, did not disturb subsequent state alternations (Fig. 12, Appendix E).

Figure 12. Even intense stimulation trains applied to the posterior hypothalamus did not abolish subsequent alternations of forebrain state.

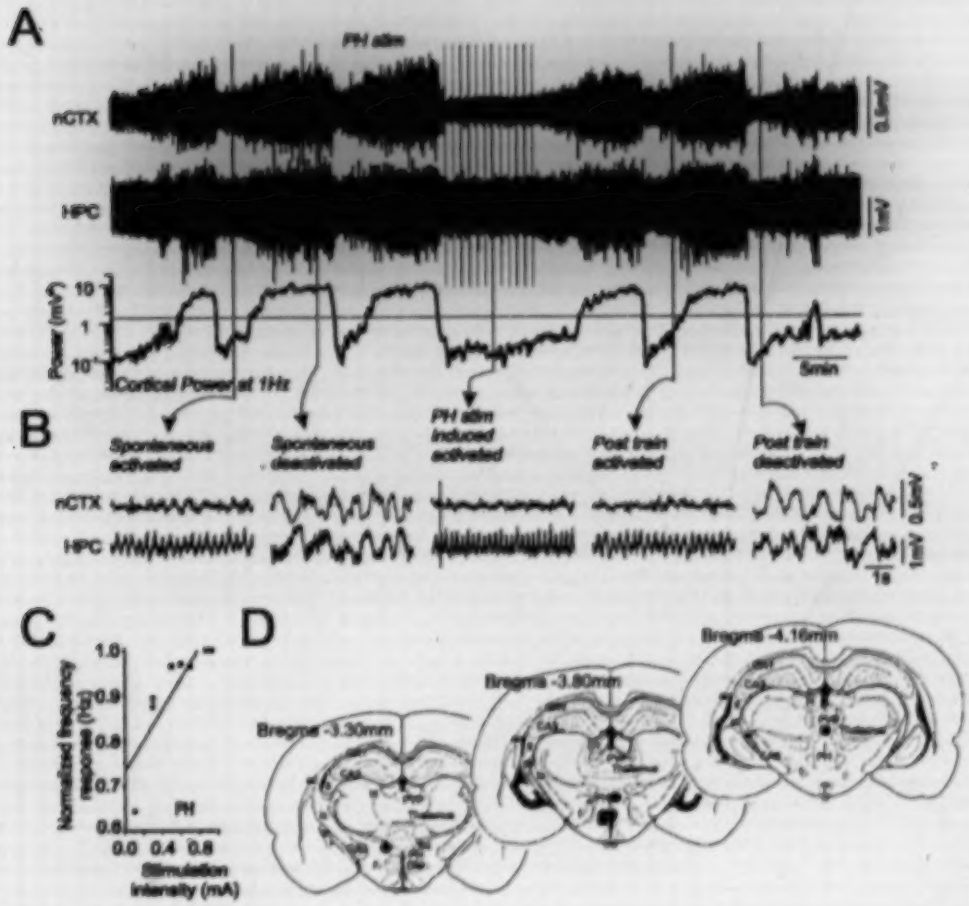

Figure 12. Even intense stimulation trains applied to the posterior hypothalamus did not abolish subsequent alternations of forebrain state.

A) Continuous EEG traces and the spectrographic cortical power at 1 Hz demonstrating the effects of stimulation of the posterior hypothalamic (PH) region. Following a stimulation train that was applied through an entire cycle, spontaneous state alternations returned to normal. **B)** Expanded EEG traces from neocortical and hippocampal sites demonstrate that activated patterns were elicited via stimulation of the PH region. **C)** Scatterplot and linear fit of frequency as a function of the stimulation intensity in the PH showing a significant ($p<0.01$) relationship between stimulation intensity and the peak frequency of theta activity recorded in the hippocampus. The frequency was normalized across experiments to the maximal frequency of theta elicited in each. **D)** Summary of histological findings for the sites of stimulation for every experiment. Abbreviations: DM: dorsal medial hypothalamic nucleus, ec: external capsule, f: fornix, fi: fimbria, H: habenular nucleus ic: internal capsule, mt: mammilothalamic tract, PH: posterior hypothalamus, PVP: paraventricular thalamic nucleus, St: stria terminalis.

Discussion

My results show that alternations of forebrain state under urethane anesthesia bear a strong resemblance in their EEG components, evolution, time course, physiological correlates and dependence upon ascending (cholinergic) activating systems to those observed during natural sleep. At a minimum, this implies that urethane induces a pharmacological condition that is highly similar to natural sleep and constitutes a good model system for the study of brain alternations during unconsciousness.

Previous studies in our own laboratory (Wolansky et al., 2006) as well as those of other researchers (Bland & Colom, 1993; Detari et al., 1997; Grahn et al., 1989; Murakami et al., 2005; Ylinen et al., 1995) have reported spontaneous fluctuations of activated and deactivated EEG patterns in animals maintained under urethane anesthesia. The present study, however, differs markedly from those conducted previously in that my recording periods were substantially longer and allowed us to describe the systematic, stereotyped and cyclic repetition of the transitions between these states. It also differs in my interpretation of these rhythmic alternations as representing a sleep-like process. Like in sleep, the transition between activated and deactivated patterns tended to be a slow process, while the opposite transition tended to be more abrupt. As well, the individual EEG characteristics of the evolution of this transition mimicked components of REM/nREM transitions through lighter and deeper slow wave stages in natural sleep. Also notable was that the timing of these periodic alternations under urethane overlapped with previous measurements of the REM/nREM cycle

(Borbely, 1976) and were nearly identical in my own experiments conducted across the two conditions in the same group of rats.

Perhaps the most convincing similarity between of state alternations across sleep and urethane was the physiological correlates. Like REM, the activated state was correlated with an increase in both heart and respiration rate. Although previous investigators (Hunter & Milsom, 1998) have attributed these changes to a continuum of arousal responses between deep and shallow anesthesia, I was unable to find any evidence equating the activated state under urethane to that evident during wakefulness (see further below). This interpretation was also at odds with my finding in a majority of animals tested in which I was able to demonstrate significant decreases in EMG tone with deactivated to activated transitions. This finding is directly comparable to the paralysis that characterizes the REM state during sleep. Interestingly, similar decreases in EMG tone with changes from deactivated to activated states under urethane have been previously reported by other groups (Horner & Kubin, 1999; Robinson et al., 1977) and can be directly observed in traces in which this difference was not systematically characterized (see Figure 2 in Hunter & Milsom, 1998).

In addition to the correlative evidence presented, I also showed that manipulations of central brain systems shown to be important for the REM/nREM sleep cycle also disrupted the expression of state alternations under urethane. Firstly, and again similarly to sleep, state alternations were entirely dependent upon muscarinic (and not monoaminergic) mechanisms. As well, the importance of ascending cholinergic inputs to cortical and hippocampal regions from the

basal forebrain that are known to influence forebrain state transitions in sleep was directly demonstrated. Finally, stimulation of brainstem sites known to be involved in the pacing of REM/nREM cycling temporarily, but potently, disrupted ongoing state alternations under urethane.

Anesthesia and sleep

The idea that anesthetics promote unconsciousness by exploiting the brain mechanisms involved in natural sleep, although popular metaphorically (Shafer, 1995), has only recently received experimental support. A number of different anesthetics have been shown to elicit their hypnotic and sedative effects through targeted pharmacological actions upon brain areas important for the elicitation of sleep (Nelson et al., 2002; Nelson et al., 2003; Tung, Bluhm, & Mendelson, 2001a; Tung, Bluhm, & Mendelson, 2001b). Furthermore, the functional physiological overlap of anesthetic action and sleep have been demonstrated in experiments that showed that animals not only avoid accruing sleep debt, but are able to recover from sleep deprivation while anesthetized (Tung, Lynch, & Mendelson, 2001; Tung & Mendelson, 2004; Tung et al., 2004), and that sleep deprived animals are more sensitive to anesthetic agents (Tung, Bluhm, & Mendelson et al., 2001b). However, it has been normally accepted that all general anesthetics produce a unitary brain state of unconsciousness similar to nREM (slow-wave) sleep (Tung & Mendelson, 2004). It is certainly the case in our laboratory that other common veterinary anesthetics at a variety of surgical anesthetic dosages do not produce the alternations apparent during urethane anesthesia (Lo, Clement, Mah, and Dickson, unpublished data). My present

results demonstrate that urethane is unique in its anesthetic action by producing a form of unconsciousness within which the expression and alternations of different sleep-like states are spontaneously exhibited. In this respect, the pharmacological action of urethane appears similar to the physiological *maintenance* of sleep rather than producing a pharmacological *induction* of a unitary sleep-like slow wave state which may constitute the action of other general anesthetics.

While urethane has been previously used as a model for the individual brain states of either nREM (including both light and slow-wave stages) or REM sleep (Contreras et al., 1997; Kinney, Vogel, & Feng, 1998; Lee, Kim, & Shin, 2004; Steriade, Nunez, & Amzica, 1993; Steriade, 1999), and while spontaneous state fluctuations have also been observed to occur with urethane anesthesia (Bland & Colom, 1993; Detari et al., 1997; Grahn et al., 1989; Murakami et al., 2005) ours is the first study to demonstrate spontaneous and rhythmic state alternations resembling those of natural sleep. My findings underscore the importance of pharmacological approaches designed to pinpoint the mechanisms by which urethane promotes its anesthetic action (Hara & Harris, 2002; Koblin, 2002; Sceniak & MacIver, 2006). Knowledge of the brain areas targeted by this drug and its effects on cellular and synaptic physiology would contribute not only to an understanding of the regulation of consciousness and of anesthetic action, but might also be relevant for the study and treatment of sleep disorders. One fruitful line of study would be to elucidate the possibility that urethane may have relatively less effect on central cholinergic mechanisms relative to other neuromodulatory systems (Vanderwolf, 1988). Certainly, my results imply that

urethane targets brain regions important for the maintenance of consciousness yet does not depress the brainstem and forebrain cholinergic mechanisms giving rise to sleep-like EEG states and their alternations.

In contrast to the interpretation of previous researchers (Detari et al., 1997; Grahn et al., 1989; Murakami et al., 2005) I found no support for the idea that the alternations present under urethane reflect fluctuations in anesthetic level. Prior studies of the metabolism of urethane have demonstrated a slow and consistent rate of decline of this substance in rodent blood samples without any evidence of systematic fluctuations across time. Indeed, in my experiments, supplemental doses of urethane did not abolish state alternations. Interestingly, and perhaps similar to the effects of sleep deprivation, these manipulations did increase the amount of time per cycle spent in the deactivated state. Furthermore, the net decrease in EMG tone observed in the majority of my EMG recordings (and the lack of change in the remainder) across deactivated to activated transitions is certainly inconsistent with a decrease in anesthetic state. Lastly, and perhaps more directly, the withdrawal threshold to painful stimuli was unchanged across both deactivated and activated states.

Related to the above, I also found no support for the contention that the activated state during urethane anesthesia resembled the waking state. It is well known that activated patterns in the forebrain during awake behavior are dependent upon a host of ascending activating systems including both cholinergic and monoaminergic components (Dringenberg & Vanderwolf, 1998; Jones, 2003; Vanderwolf, 1988). Monoaminergic depletions using reserpine, whether

conducted prior or during anesthesia, were without effect upon the activated state, and indeed, upon the presence of state alternations. Consistent with these findings, the activated state (and moreover state alternations) during urethane anesthesia was completely abolished by muscarinic receptor antagonism – similar to effects reported for the naturally sleeping animal.

Despite the similarities reported here, there are some obvious differences between urethane anesthesia and sleep. Firstly, (and as previously acknowledged (Tung & Mendelson, 2004)), sleep and its states are homeostatically regulated, and therefore internally driven. In contrast, the anesthetized condition was dependent upon circulating levels of exogenously derived urethane. Moreover, anesthesia (by definition) is a condition from which an organism cannot be "awakened" until the circulating anesthetic agents have been metabolized. Secondly, the hallmark signs of REM sleep, rapid-eye movements, were absent in the transitions from deactivated to activated patterns of brain state under urethane anesthesia. Indeed, a related dissimilarity was the open eyelid posture which was maintained under urethane. This, along with other minor differences, suggests (not surprisingly) that the absolute spectrum of physiological changes present during natural sleep cycles is not completely mimicked by urethane anesthesia. However, both the similarities and differences between the urethane and sleeping conditions provide an intriguing means to elaborate the central and peripheral mechanisms involved in the constellation of the physiological correlates of state alternations during unconsciousness themselves.

Although the mechanisms by which state alternations took place under urethane anesthesia were not directly examined in the present study, the importance of brainstem nuclei known to be involved in sleep cycle alternations was emphasized. I was able to robustly interfere with ongoing state alternations with relatively moderate trains of electrical stimulation of sites in the region of the PPT. Although forebrain activation was a prominent effect during stimulation trains, the effect subsequent to a series of trains was a pronounced and lasting deactivation of forebrain regions. Although state alternations were abolished for a variable period following brainstem stimulation, they spontaneously returned. This suggests that, like in sleep, these brainstem regions form part of an activity dependent circuit pacing the alternations themselves and that exogenous activation can disrupt the balance of these different elements. It is notable in this regard, that even prolonged and strong stimulation trains applied to more rostral brain sites such as the posterior hypothalamus (which form part of the ascending activating system but is not thought to be involved in pacing state alternations during sleep *per se*) caused no such temporary depression of the ongoing alternations although they did profoundly induce forebrain activation during applied trains. These findings define an interesting avenue for future study of the mechanisms involved in state transitions under urethane.

Based upon these data, I consider that urethane anesthesia constitutes a viable and realistic model system for the alternations of brain state present during natural sleep. This model will undoubtedly aid mechanistic investigations and lead to

insights concerning the functional relevance of fluctuations of brain state during unconsciousness. Furthermore, it will potentially facilitate the development of novel sleep agents designed to induce and maintain behavioral unconsciousness without disrupting the brain's natural tendency to fluctuate between different states. The advantage of studying these dynamic events in an anesthetized preparation, which allows for unfettered control and access, will clearly benefit the field of sleep research and, indeed, neuroscience in general.

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Chapter 3 – General Discussion

Summary

This study investigated the novel finding of cyclic alternations of brain state in the urethane-anesthetized animal and evaluated their resemblance to alternations seen in natural sleep. Similarities were found between these two conditions in the spectral properties of states, in the time course of alternations and in the physiological correlates known to fluctuate across the REM/nREM sleep cycle. It further demonstrated the similarity across conditions in the underlying neurotransmitter systems and brain mechanisms involved in states and state transitions.

Evaluation of the urethane-anesthetized model of natural sleep

Applicability of the urethane-anesthetized model in the study to natural sleep

The use of the urethane-anesthetized model will facilitate investigations into brain states similar to those in sleep, including electrographic correlates of both activated (REM sleep-like) and deactivated (nREM sleep-like) patterns. Additionally, recordings and investigations of sleep-related areas, such as the brainstem, hypothalamus and basal forebrain, across the activated/deactivated cycle are likely to prove useful in elucidating mechanisms of these states. Furthermore, investigations into rhythms that are associated with transitions into slow-wave sleep, such as the slow (<1Hz) oscillation and sleep spindles, are also

less likely to be hindered. There is potential, as well, for further study of the mechanisms underlying physiological correlates, including respiration, heart rate, and the mechanisms contributing to REM muscle atonia. This model can be used to clarify the roles of specific neurotransmitters in both sleep states and alternations, and to elucidate mechanisms underlying transitions into and out of REM sleep, perhaps clearing up some of the debate surrounding, for example, the loci and interactions involved in state transitions. Furthermore, focus on the electrographic properties of structures that are implicated in memory processing during sleep, such as the hippocampus and the cortex, and how their interactions vary with state changes, could offer possible mechanisms that are being used in the consolidation of memory.

Absence of brain state alternations in other veterinary anesthetics

The applicability of urethane to sleep is further highlighted by experiments that investigated brain rhythms under other common veterinary anesthetics, such as pentobarbital, ketamine-xylazine, and isoflurane, under all of which brain states were unitary, demonstrating that alternations of brain state are, in fact, exclusive to urethane. Both pentobarbital and isoflurane induce anesthesia largely via potentiation of the inhibitory neurotransmitter GABA (Rudolph & Antkowiak, 2004). Ketamine-xylazine, on the other hand, is a veterinary anesthetic believed to induce its sedative affects via antagonism of NMDA receptors, and agonism of noradrenergic receptors (Greene & Thurmon, 1988; Harrison & Simmonds, 1985; Rudolph & Antkowiak, 2004). Under ketamine-xylazine anesthesia, a prominent

slow oscillation was apparent in both the cortex and hippocampus, and long duration recordings demonstrated an absence of alternations of state (Lo, Clement, Mah, Dickson, unpublished data). Under pentobarbital and isoflurane, animals displayed cortical and hippocampal burst suppression, sometimes characterized by long durations of electrical silence in their activity. Here, again, there was an absence of rhythmic alternations, as was seen under both urethane anesthesia and in natural sleep. Any changes in brain state observed under all three anesthetics were concomitant with loss of anesthesia, whereas anesthetic state was consistent across alternations under urethane. These results support the idea that urethane anesthesia is unique, increasing its relevance for sleep research.

Limitations of the urethane-anesthetized model of natural sleep

Despite the versatility of urethane anesthesia in the study of sleep, like all models, there are limitations. Firstly, urethane, as described above, is a toxic substance, and its use as a veterinary anesthetic is limited to acute experiments. This essentially means that urethane is not suitable for extensive behavioural experiments where the manipulations under anesthetic are followed by performance testing. Secondly, there was a lack of EOG activity, which may limit research into the physiology of rapid-eye movements. As urethane is known to produce atonia itself, this finding is perhaps not surprising (Robinson, Kramis, & Vanderwolf, 1977). What was surprising, therefore, was that in the majority of the animals with functional EMGs, declines in tone were observed with transitions into the activated state, as would be observed in natural sleep. This implicates the usefulness of the urethane-anesthetized animal in the study of mechanisms that

underlie the REM sleep muscle atonia, an area of research with much clinical relevance.

Potential relevance of the urethane-anesthetized animal in the treatment of sleep related disorders

Potential relevance to insomnia

The clinical relevance of the urethane-anesthetized animal includes the treatment of insomnia, a disorder that is characterized by the inability to initiate or maintain sleep, or lack of restorative sleep, for a minimum duration of one month (Mahowald & Schenck, 2005; Rosenberg, 2006). It is estimated that insomnia affects 10 to 15% of people chronically and another 25-35% transiently (Rosenberg, 2006). Most treatments for insomnia compromise next day functioning, which could be related to their promotion of slow-wave sleep at the expense of REM (Rosenberg, 2006). To circumvent these issues, sleep agents with shorter half-lives have been developed, however this can result in sleep maintenance insomnia, where night-time awakenings are followed by the inability to re-enter the state of sleep. Sleep maintenance insomnia is also prevalent among specific populations, including the elderly and those with pain syndromes (Rosenberg, 2006). An effective sleep agent, one which would offer relief of both sleep onset and sleep maintenance insomnia without compromising next day functioning, would be of immense value. Given the spectrum of sleep that was mimicked under urethane anesthesia, especially alternations of brain state,

urethane could provide a molecular gateway to the discovery of new drugs, helping to address these major issues.

Furthermore, the urethane-anesthetized model could also be a useful tool in testing potential sleep agents as well as drugs which have side-effects that compromise sleep. Because it is known that sleep agents often disrupt the cyclicity of the REM/nREM sleep cycle, use of this model could provide insight into the feasibility of new drugs, by evaluating their effect on state alternations. Moreover, drugs which are known to compromise sleep, as a side-effect, could be evaluated for the relevant potential mechanisms, either leading to an increased understanding of sleep, or to the production of a newer drug which excludes such aversive side-effects.

Support for this claim has come from recent experiments using the administration of anti-histaminergic drugs used to treat allergies. These drugs are traditionally associated with somnolence, however newer anti-histamines have been produced and marketed for the purpose of circumventing this aversive side effect (Jones, 2005; Montoro et al., 2006). When a first generation anti-histamine was administered to an animal anesthetized with urethane, the appearance of brain states was altered similarly as they would be in natural sleep (Goulko, Cassault, and Dickson, unpublished data, (Monti, Pellejero, & Jantos, 1986). In contrast, administration of a second generation anti-histamine resulted in no significant effect on states seen under urethane. These results strengthen the potential of the urethane-anesthetized animal as a model of natural sleep.

Potential relevance to disorders of REM sleep

The clinical relevance of the urethane-anesthetized animal extends beyond insomnia to include disorders like narcolepsy and REM behaviour disorder. These disorders often have dissociations of REM sleep and REM sleep muscle atonia, where one is present in the absence of the other. Narcolepsy is characterized by involuntary transitions from wake to REM sleep despite having had sufficient rest the previous night (Mahowald & Schenck, 2005). Conversely, REM behaviour disorder is characterized by a lack of muscle atonia during REM sleep, which results in the affected individual acting out their dreams, which are frequently associated with violence, resulting in either personal injury or the injury of a bedmate (Mahowald & Schenck, 2005; Paparrigopoulos, 2005). Recently, it was found that orexinergic neurons of the lateral hypothalamus are absent in narcoleptics (Chemelli et al., 1999). Regardless, it remains unclear exactly how the absence of these neurons contributes to the induction of REM sleep complete with muscle paralysis on the background of wakefulness. And although brainstem pontine regions have been implicated in REM sleep associated atonia, it remains unclear how atonia is, in fact, induced during REM sleep (Jones, 2005; Paparrigopoulos, 2005). Animal models of these disorders under urethane anesthesia could provide major advantages into the investigations of REM sleep muscle atonia, as well as into the mechanisms of sleep onset and state transitions. Furthermore, the urethane-anesthetized animal could provide a method for elucidating the mechanisms that are involved in muscle atonia during REM sleep, which could have clinical relevance in terms of treatment of these disorders.

Concluding remarks

It is apparent that sleep presents a great deal of complexities and despite major advances and continued efforts in the research community, our understanding of sleep remains incomplete. With sleep deprivation costing the US government an estimated $15 billion in health care and an additional $50 billion in lost work hours and productivity, it becomes clear that this lack of understanding could contribute to the ineffectiveness of clinical treatments (National Institutes of Health, 2004). Increased study of sleep is essential, however, it is hindered by the lack of a model that represents the full spectrum of natural sleep. Ultimately, this study demonstrated that the urethane-anesthetized animal shows a great deal of promise in terms of bridging the gaps in current sleep research.

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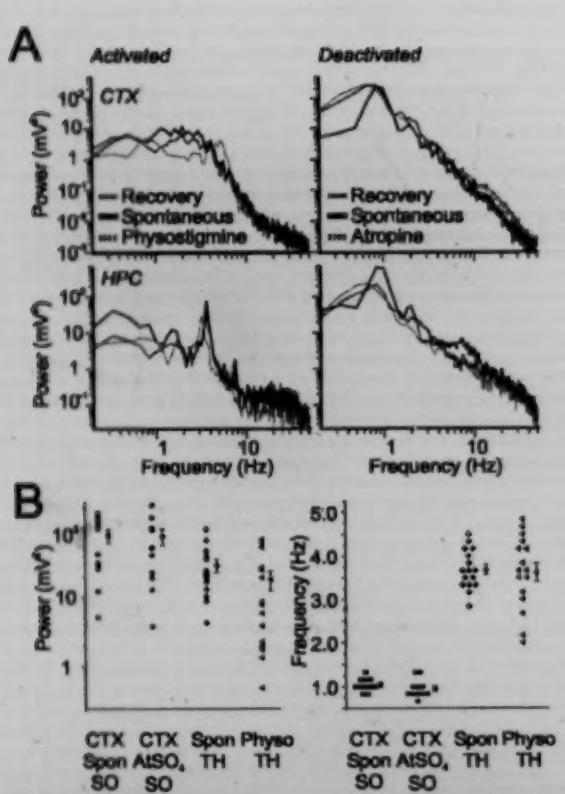
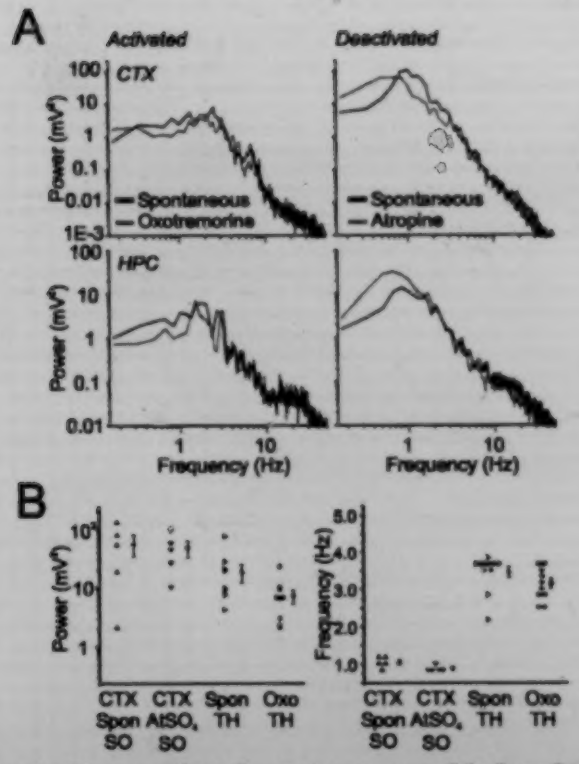


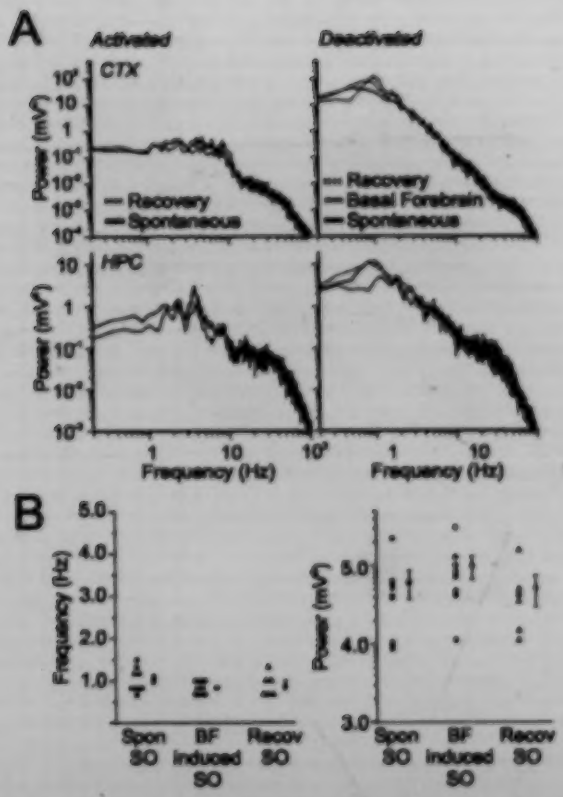
Appendix A. Spectral properties of spontaneous and induced neocortical and hippocampal states are similar with manipulations using physostigmine and atropine.

A) Neocortical and hippocampal spectra for spontaneous and cholinergically induced activated and deactivated states. As with the raw EEG (see Figure 7), spontaneous and induced states are highly similar. **B)** Peak powers and frequencies for all spontaneous and induced brain states. All pair-wise comparisons were not significantly different except for the peak power of hippocampal theta which was lower during physostigmine treatments ($p=0.004$).



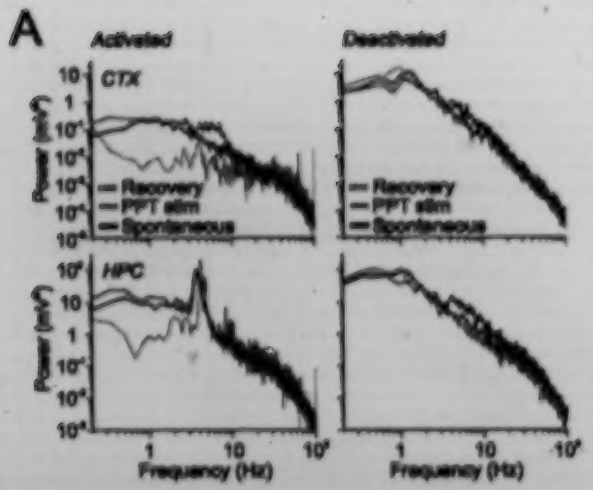
Appendix B. Spectral properties of spontaneous and induced neocortical and hippocampal states are similar with manipulations using oxotremorine and atropine.

A) Neocortical and hippocampal spectra for spontaneous and muscarinic-receptor stimulation induced activated and deactivated states. As with the raw EEG (see Figure 8), spontaneous and induced states were highly similar. B) Peak powers and frequencies for spontaneous and induced brain states. All pair-wise comparisons were not significantly different except for the peak power of hippocampal theta which was lower during oxotremorine treatment ($p=0.04$) and the peak frequency of the cortical slow oscillation which was lower during atropine treatments ($p=0.03$).



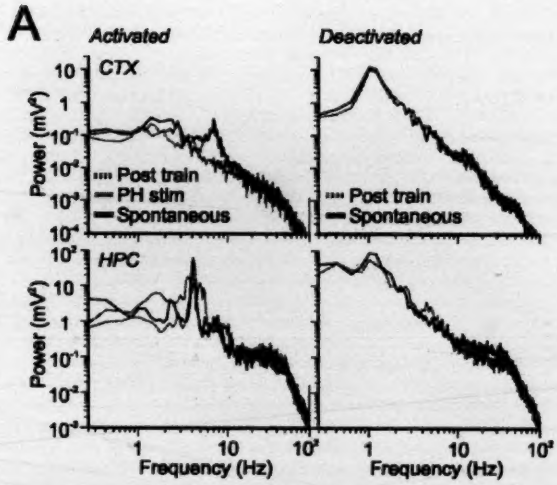
Appendix C. Spectral properties of spontaneous and basal forebrain inactivation induced neocortical and hippocampal deactivated states are similar.

A) Neocortical and hippocampal spectra for spontaneous and basal forebrain inactivation induced deactivated states. As with the raw EEG (see Figure 10), spontaneous and induced states are highly similar. B) Scatterplot representing peak power and frequencies of spontaneous, basal forebrain inactivation induced and recovery cortical slow oscillation. Although there were no significant differences in peak frequency ($p>0.05$), peak power of the basal forebrain inactivated slow oscillation was significantly higher than spontaneous and recovery slow oscillation ($p<0.05$).



Appendix D. Spectral properties of activated EEG patterns are similar across spontaneous and PPT-stimulation induced conditions.

A) Neocortical and hippocampal spectra for states occurring spontaneously and with stimulation of the pedunculo-pontine tegmentum (PPT). As with the raw EEG (see Figure 11), spontaneous and induced states appear highly similar.



Appendix E. Spectral properties of activated EEG patterns are similar across spontaneous and PH-stimulation induced conditions.

A) Neocortical and hippocampal spectra for states occurring spontaneously and with stimulation of the posterior hypothalamus (PH). As with the raw EEG (see Figure 12), spontaneous and induced activated states were highly similar.